

CELL WALL METABOLISM IN RIPENING AND CHILLED TOMATO FRUIT AS
INFLUENCED BY THE pH AND MINERAL COMPOSITION OF THE APOPLAST

By

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LIST OF ABBREVIATIONS

β -Gal	β -galactosidase
BP	buffered phenol
CDTA	trans-1,2-cyclohexylenediamine- <i>N,N,N'</i> -tetraacetic acid
DP	degree of polymerization
EIS	ethanol insoluble solids
HEPES	N-2-hydroxyethylpiperazine- <i>N'</i> -2-ethane sulfonic acid
MCP	1-methylcyclopropene
PG	polygalacturonase
PME	pectinmethylesterase

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Cell wall metabolism in ripening fruit is likely to be influenced by the pH and ionic properties of the apoplast. The objectives of the research reported in this dissertation were to characterize the pH and mineral composition of ripening tomato fruit and to determine their effect on polygalacturonase (PG, EC 3.2.1.15) catalysis. Pectin solubilization and depolymerization was studied in ripening and chill-injured fruit using transgenic fruit with down-regulated PG.

Pectin solubilization *in vivo*, as determined by the analysis of apoplastic exudates and pectin efflux from pericarp disks, was very restricted and independent of polygalacturonase activity. In chill-injured mature-green fruit, pectin solubilization remained at the levels found before low-temperature storage, despite decreased firmness,

indicating that chilling-induced softening is independent of pectin solubilization and depolymerization.

The apoplastic solution of mature-green fruit had pH near 7 and acidified during ripening to pH 4.5 in ripe fruit. Apoplastic potassium levels increased 3-fold during ripening, from 13 mM in mature-green fruit to 37 mM in fully ripe fruit. Calcium and magnesium levels remained relatively constant during ripening at 5 and 9-13 mM, respectively. Low-temperature storage induced acidification of the apoplast, but mineral levels remained constant during cold storage. Transfer to higher temperature following cold storage resulted in decreased levels of apoplastic cations.

PG activity was very restricted in solutions mimicking the composition of the apoplastic fluid of ripening tomato, in contrast with high ionic strength, calcium-free buffers. PG was able to hydrolyze ionically bound (CDTA-soluble) pectins.

Pectinmethylesterase reduced uronic acid solubilization from cell walls.

It is concluded that apoplastic pH and mineral composition provide a strong means for regulating cell wall metabolism in ripening and chill-injured fruit. It is proposed that the dynamic changes in the composition of the apoplastic solution during fruit ripening provide a biochemical regulatory mechanism, in addition to the transcriptional regulation, for the sequential action of hydrolases that disassemble cell walls and cause fruit softening.

CHAPTER 1

INTRODUCTION

During ripening, fleshy fruits undergo textural changes that affect their quality, marketability, harvest, and postharvest handling techniques. Ripening-related softening is associated with cell wall metabolism. A number of enzymes have been implicated in cell wall metabolism in ripening fruit, including polygalacturonase, pectinmethylesterase, β -galactosidase, xyloglucan endotransglycosylase, endo- β -1,4-glucanase and expansin. Although these enzymes have been the subject of many biochemical and molecular studies, a unifying model explaining how several enzymes act together to bring about the changes in cell wall polysaccharides associated with fruit softening is still lacking.

There are several examples of discrepancies between the *in vitro* potential of cell wall hydrolases and their effects *in vivo*. Evidence for *in vivo* limitations of activity have been presented for polygalacturonase (Seymour et al. 1987) and β -galactosidase activity *in vitro* seems to be much less than necessary for the observed losses of galactose *in vivo* (De Veau et al., 1993; Ross et al. 1993). Moreover, down-regulation of polygalacturonase (Smith et al., 1988), pectinmethylesterase (Tieman and Handa, 1994), and endo- β -1,4-glucanases (Lashbrook et al, 1998; Brummell et al., 1999) by antisense technology has shown that individual enzymes have little effect on the initial stages of fruit softening. Cell wall enzymes are strongly affected by pH, minerals, and ionic strength. Nevertheless, the apoplastic pH and mineral composition of ripening fruit have received little attention.

The objectives of the research described herein were the following: to characterize the pH and mineral composition of the apoplastic solution of ripening tomato; to determine the activity of polygalacturonase in solutions mimicking the apoplastic conditions, in contrast with conventional buffers; to compare pectin solubilization in vivo and in vitro; to determine apoplastic conditions and pectin metabolism in chilling-injured fruit.

CHAPTER 2 LITERATURE REVIEW

Introduction

Ripening is a developmental process that makes fruit fit for their function of seed dispersal and species survival. Like all senescence phenomena, fruit ripening is determined by the internal metabolism of the fruit and leads, irreversibly, to organ death. This process is of immense horticultural importance. Ripening generally renders the fruit edible, except in the cases of commodities where horticultural maturity is attained before ripening (e.g., cucumber, okra). Fruit ripening involves changes in several biochemical pathways, originating a complex syndrome. At the physiological (organ) level, the most evident alterations involve color changes brought about by plastid and vacuole senescence, alterations in composition and volatiles, causing changes in flavor and aroma, and softening. In climacteric fruit these alterations are usually, but not necessarily, synchronized and coordinated by ethylene.

Fruit softening is a major aspect of the ripening syndrome in many fruits and has enormous horticultural relevance. Softening increases susceptibility to bruising and decay, imposing limitations to handling practices, storage, and shipment duration. Softening is considered to be a consequence of cell wall degradation. Cell wall disassembly is a central process in plant biology. The most extreme forms of cell wall

degradation occur in phenomena that involve cell-cell separation, including organ abscission and anther dehiscence, penetration of the pollen tube in the pistil, seed germination, and the ripening of fleshy fruit. As all these processes share some basic characteristics, cell wall metabolism in ripening fruit can provide a model for the understanding of the general mechanism of cell wall disassembly.

Correlative evidence has implicated several cell wall enzymes in fruit softening. For the most widely studied cell wall hydrolases there are instances where gene expression and extractable enzyme activity correlate well with the softening pattern; however, a close examination of the data frequently show weakness in the correlation. No single enzyme can account for the scope of changes in the cell wall of ripening fruit, and it is likely that no one enzyme can bring about fruit softening in all fleshy fruits. Instead, softening is probably caused by the concerted action of several enzymes in the wall structure.

This review examines the patterns of gene expression and biochemical regulation of activity of the cell wall enzymes and highlights the potential for regulation of cell wall disassembly by the apoplastic pH and mineral composition, in an attempt to understand how the activity of different enzymes may be integrated to bring about cell wall disassembly in ripening fruit.

Cell Wall Changes during Ripening

Cell walls are extremely complex and, despite a wide array of chemical and physical techniques, continue to defy structural elucidation. The primary cell wall is composed of polysaccharides, structural proteins, and a number of nonstructural

components, including enzymes. Current models envision the cell wall as being composed of three coextensive networks: the cellulose-hemicellulose framework, the pectic matrix, and a network of structural proteins (Carpita and Gibeaut, 1993).

Cellulose, a linear chain of β -1 $\rightarrow$ 4-D-glucosyl residues, is deposited in the wall as microfibrils 5 to 15 nm in diameter and accounts for one-fourth to one-third of total wall polysaccharides (Carpita and Gibeaut, 1993; Reiter, 1994). The microfibrils are linked by xyloglucans, the major hemicellulose in dicots. Xyloglucans are heteropolymers composed of a linear chain of β -1 $\rightarrow$ 4-D-glucosyl residues with α -D-xylosyl residues attached to the O-6 position of most glucosyl residues (Reiter, 1994). Some of the xylose units are further modified at position 2 by the attachment of β -D-galactosyl, α -L-arabinosyl, or α -L-fucosyl-1 $\rightarrow$ 2- β -D-galactosyl units (Carpita and Gibeaut, 1993; Reiter, 1994). Xyloglucan polymers bind strongly to cellulose by hydrogen bonding but exhibit weak interpolymeric interactions.

The cellulose-xyloglucan framework is coextensive with the pectic matrix, which accounts for about one-third of the primary cell wall in dicots (Carpita and Gibeaut, 1993; Reiter, 1994). The major pectic polysaccharides are homogalacturonan (HG), helical homopolymers of 1 $\rightarrow$ 4- α -D-galactosyluronic acid, and rhamnogalacturonan I (RG I), heteropolymers with a repeating structure of 1 $\rightarrow$ 2- α -L-rhamnosyl-1 $\rightarrow$ 4- α -D-galactosyluronic acid. A highly branched and compositionally diverse rhamnogalacturonan II (RG II) is found in small amounts.

Research on cell wall metabolism during fruit ripening has focused on characterizing the physical and chemical alterations in the various polysaccharides and identifying and characterizing the enzymic activities that bring about these changes.

Since the topic of cell wall hydrolases was last the object of major reviews (Fischer and Bennett, 1991; Huber, 1983a), a great deal has been learned about their transcriptional regulation, two other enzymes—XET and expansin—have been identified, and β -galactosidase/galactanase have received increased attention. Even so, a comprehensive model for the integration of all the enzymes in cell wall disassembly is still lacking. The next sections briefly review the alterations of wall polysaccharides during fruit ripening, the putative enzymes involved, and introduce the idea that changes in the activity of cell wall hydrolases may be caused by alterations in the apoplastic environment.

Cellulose-Xyloglucan Framework

The cellulose-hemicellulose framework undergoes alterations during ripening, mainly due to hydrolysis of hemicelluloses and possibly the disruption of the noncovalent bonds between cellulose and xyloglucan. Most evidence suggests that cellulose levels remain constant during fruit ripening (Bartley, 1976; Ahmed and Labavitch, 1980; Maclachlan and Brady, 1994; Nunan et al., 1998), despite occasional reports of decreases (Sakurai and Nevins, 1993). No depolymerization of cellulose is observed during ripening of tomato pericarp, but limited breakdown occurs in the locule tissue (Maclachlan and Brady, 1994).

Altered hemicellulose metabolism is active in ripening fruit. Hemicelluloses are increasingly solubilized, depolymerized and debranched during ripening, although to different extents among fruit types. The proportion of hemicellulose in persimmon fruit cell walls decreases during fruit growth and ripening (Cutillas-Iturralde et al., 1994). In contrast, levels of xyloglucan remain constant during ripening of normal tomato fruit but

decrease slightly during senescence of *rin* fruit (Maclachlan and Brady, 1994). The molecular mass distribution of hemicellulose changes during ripening of several fleshy fruit including tomato pericarp (Huber, 1983b), muskmelon (McCollum et al., 1989), strawberry (Huber, 1984) and persimmon (Cutillas-Iturralde et al., 1994).

Hemicelluloses undergo deglycosylation during ripening. Reduction in the content of xylose and galactose was reported for the hemicelluloses of ripening melon (Rose et al., 1998). Persimmon fruit hemicelluloses lose arabinose, galactose, and glucose during growth and ripening. In this fruit, xylose levels in the hemicellulose fractions decrease during fruit growth, but not during ripening (Cutillas-Iturralde et al., 1994). Most studies about the ripening-related loss of neutral sugars from cell wall polysaccharides fail to determine the specific origin of the released sugars. Similarly, it is not clear what are the preferred substrates *in vivo* for the most widely studied deglycosylase present in fruit, namely β -galactosidase/galactanase. This enzyme will be discussed in connection with the disassembly of the pectic network, noting however, that hemicellulosic side chains possess β -D-galactopyranosyl residues that could be targeted by this enzyme.

Enzymes that Impact the Cellulose-Hemicellulose Network

Two enzymes are capable of depolymerizing xyloglucan: endo- β -1,4-glucanases and xyloglucan endotransglycosylase. Additionally, expansins are thought to disrupt hydrogen bonding between xyloglucan and cellulose. Other enzymes contribute to the deglycosylation of xyloglucan side chains.

Xyloglucan endotransglycosylases

Xyloglucan endotransglycosylase (XET; EC 2.4.1.-) cleaves xyloglucan and transfers part of the polymer to the non-reducing end of other xyloglucan fragments. This enzyme was first identified in vegetative organs of several plants (Fry et al., 1992) and purified from an apoplastic extract from *Vigna angularis* epicotyls (Nishitani and Tominaga, 1992). Redgwell and Fry (1993) reported XET activity in ripening kiwifruit, and fruit-expressed genes have been cloned in tomato (de Silva et al., 1994; Arrowsmith and de Silva, 1995) and kiwifruit (Schröder et al., 1998). XET activity has been reported in kiwifruit (Redgwell and Fry, 1993; Percy et al., 1996; Vincken et al., 1998), and in tomato (Maclachlan and Bray, 1994), persimmon (Cutillas-Iturralde et al., 1994) and apple (Percy et al., 1996).

Activity against native xyloglucan was demonstrated for kiwifruit XET (Redgwell and Fry, 1993). Kiwifruit XET has both transglycosylase and hydrolase activities (Schröder et al., 1998), but the tomato enzyme appears to have only transglycosylase activity (de Silva et al., 1994; Arrowsmith and de Silva, 1995).

XET activity has been reported to increase during ripening of tomato (Maclachlan and Brady, 1994), kiwifruit (Redgwell and Fry, 1993; Percy et al., 1996; Vincken et al., 1998) and persimmon (Cutillas-Iturralde et al., 1994) fruits. In apple fruit, XET activity has been reported to increase (Vincken et al., 1998) or decrease (Percy et al., 1996). Although Maclachlan and Brady (1992) did not unequivocally assay for XET activity, the "oligosaccharide-activated xyloglucanase" activity they observed in tomato pericarp increased from the mature-green to the pink stage, and then decreased at the ripe stage. de Silva et al. (1994) reported a decrease in extractable XET activity from green to

turning tomato fruits despite an increase in the levels of mRNA for tXET-B1, an isoform present in breaker fruit. This may be attributed to differential patterns of accumulation of XET isozymes. In fact, in tomato pericarp, buffer-soluble XET increased during ripening, whereas buffer-insoluble XET decreased resulting in only a slight increase in total XET activity (Maclachlan and Brady, 1994).

Endo-1,4- β -glucanases

Endo-1,4- β -glucanases (EGase, EC 3.2.1.4) catalyze the hydrolysis of β -1 $\rightarrow$ 4-glucosyl linkages. However, EGase activity is assayed by measuring the ability of the enzyme to degrade the artificial soluble cellulose derivative carboxymethylcellulose (CMC), and doubts persist about the *in vivo* substrate for plant EGases. Sobotka and Stelzig (1974) reported on a complex of four enzymes in tomato that were capable of *in vitro* degradation of insoluble cellulose. In contrast, avocado EGase showed little or no activity against cellulose (Hatfield and Nevins, 1986), and the enzyme exhibits little capacity to degrade avocado xyloglucan (O'Donoghue and Huber, 1992). Other authors could not detect activity of tomato EGase against insoluble cellulose (Pharr and Dickinson, 1973), but O'Donoghue et al. (1994) have suggested that noncrystalline regions of cellulose may be targeted by the enzyme. The avocado enzyme does not hydrolyze cellobiose and prefers celloextrins with a degree of polymerization higher than 4 (Hatfield and Nevins, 1986). However, tomato fruit have cellobiases (EC 3.2.1.21) capable of degrading β -1,4-disaccharides (Pharr and Dickinson, 1973; Sobotka and Stelzig, 1974).

Typically, EGase activity decreases during fruit growth (Hobson, 1968; Bonghi et al., 1998). From minimum activity levels at the pre-climacteric stage, EGase activity rises during ripening to a maximum at or just before the ripe stage, at which stage a decline in activity is frequently observed. This trend has been reported for tomato (Hall, 1964; Sobotka and Watada, 1971; Huber, 1985), peach (Bonghi et al., 1998), and avocado (Pesis et al., 1978; Awad and Young, 1979; Christoffersen et al., 1984) fruits. In avocado, EGase protein is present at very low levels in preclimacteric fruit and accumulates during ripening in parallel with increased EGase activity (Christoffersen et al., 1984). The levels of protein in the endoplasmic reticulum diminish after the climacteric peak, indicating diminished secretion (Dallman et al., 1989).

Regulation of Cellulose-Xyloglucan Framework Disassembly in Ripening Fruit

Metabolism of the cellulose-hemicellulose framework during fruit ripening has received much attention in recent years, prompted by the work with transgenic tomato (Smith et al., 1988; Giovannoni et al., 1989) suggesting that pectin depolymerization was not the sole factor responsible for fruit softening. Correlative evidence between EGase activity and fruit softening is inconclusive (e.g., Sobotka and Watada, 1971; Hobson, 1968). However, the developmental regulation of EGase genes during ripening (Lashbrook et al., 1994; Gonzalez-Bosch et al., 1996; Llop-Tous et al., 1999) and the evidence that xyloglucan metabolism precedes the breakdown of the pectic matrix (Rose et al., 1998) may implicate the disassembly of the cellulose-xyloglucan network in the early stages of fruit ripening.

The next section provides a review of the transcriptional and biochemical regulation of the enzymes likely to contribute to the disassembly of the cellulose-xyloglucan framework in ripening fruit, and attempts to integrate their expression patterns and their pH-activity profiles to understand the changes observed in the cellulose-xyloglucan framework during ripening.

Transcriptional Regulation

The expression of cell wall hydrolase genes is under hormonal and developmental regulation. Ethylene induces the expression of fruit expansin (Rose et al., 1997), EGase (Lashbrook et al., 1994; Gonzalez-Bosch et al., 1996) and XET (Schröder et al., 1998). The promoter of the avocado *cell1* gene, encoding for a fruit EGase, contains sequences similar to several ethylene-inducible genes (Cass et al., 1990). However, developmental cues alone may be sufficient to regulate the expression of some of these genes. In fact, the expression pattern of homologue EGase genes during ripening of climacteric and nonclimacteric fruit is similar as shown by the expression of strawberry *Cel1* (Llop-Tous et al., 1999) and tomato *Cel2* (Lashbrook et al., 1994).

The expression of ripening-related genes encoding enzymes capable of acting upon the cellulose-xyloglucan framework is depicted in Figure 2-1. The figure was derived from data for the expression of tomato EGase (Lashbrook et al., 1994; Gonzalez-Bosch et al., 1996), XET (Arrowsmith and de Silva, 1995) and expansin (Rose et al., 1997) genes. Due to differences in units used to report levels of mRNA, they are presented as a percentage of maximum transcript levels found in fruit.

In mature-green fruit, only XET is expressed at significant levels, about 40% of the maximum levels found at the pink stage. This is in agreement with the suggestion that XET-mediated cell wall changes may be an early step in modifying the cell wall, rendering it more susceptible to degradation by other hydrolases (Redgwell and Fry, 1993). XET activity, however, requires the presence of xyloglucan oligosaccharides (Fry et al., 1992). These could be generated by XET of the type characterized for kiwifruit, which possesses hydrolase activity (Schröder et al., 1998), but not by tomato XET, that appears to have only transglycosylase activity (de Silva et al., 1994; Arrowsmith and de Silva, 1995). Alternatively, the oligosaccharides could be generated by EGases.

Figure 2-1 requires further explanation in relation to the expression of EGases. Cel2 is the major EGase in ripe tomato fruit and Cel1 mRNA is present at comparatively low levels. In absolute terms, both Cel1 and Cel2 mRNA are present at similar levels in breaker through pink fruit. Between the pink and overripe stages Cel2 increases more than 15-fold while levels of Cel1 remain low. However, when expressed as a percentage of maximum expression, it become evident that EGase expression spans from the breaker to the overripe stages, with Cel1 contributing most of its total relative activity at the breaker-turning stages and Cel2 contributing more in ripe fruit. Maclachlan and Brady (1992) detected 3 EGase activities in tomato, one with optimum activity at pH 5 and two with optimum activity at pH 7. It is interesting to note that although both activities (pH 5 and 7) increase during ripening, the pH 7 EGase activity increases about 3-fold whereas the pH 5 increases about 2-fold and shows an increase during the latter stages of ripening (Maclachlan and Brady, 1994). These two EGase activities must be taken into consideration when discussing the role of apoplastic pH on cell wall metabolism. The

three isozymes may be products of different genes, or products of a single gene modified post-translationally or post-transcriptionally.

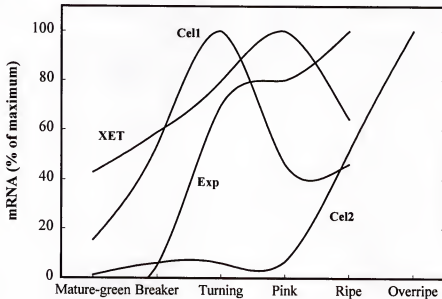


Figure 2-1. Expression of tomato EGase (Cell and Cel2), XET and expansin (Exp) genes during ripening. Data derived from Lashbrook et al. (1994) and Gonzalez-Bosch et al. (1996) (EGase), Arrowsmith and de Silva (1995) (XET) and Rose et al. (1997) (expansin).

Based on the gel blots presented by Rose et al. (1997), the pattern of expression of the expansin gene (*LeExp1*) is tentative, because the authors gave no quantitative information about the transcript levels. The gene is not expressed in mature-green fruit and there is a large increase in expression from the breaker to the turning stages and a subsequent increase at the ripe stage. The authors suggest that expansin may play a role in cell wall degradation during ripening by disrupting non-covalent interactions in the cellulose-hemicellulose network, or alternatively in the hemicellulose-pectin network.

The disruption of these networks would favor cell wall disassembly by exposing previously inaccessible substrates to hydrolases or transglycosylases. Expansin expression patterns, however, are not informative about the mode of action of the fruit enzyme.

Regulation of Cellulose-Xyloglucan Disassembly by pH and Minerals

Figure 2-2 shows the effect of pH on the activity of enzymes implicated in the disruption of the cellulose-xyloglucan network. In the absence of detailed information about the tomato enzymes, the data for the *Arabidopsis* XET TCH4 (Purugganan et al., 1997), peach EGase (Hinton and Pressey, 1974) and two expansins (McQueen-Mason et al., 1992; Li et al., 1993) were utilized. *Arabidopsis* XET TCH4 was chosen because of its strong sequence homology with tomato tXET-B1 (Nishitani, 1997). The chart suggests that the concerted action of these 3 enzymes may impact the cellulose-xyloglucan network over the pH range 3.5-7.5.

The depicted pH-activity profile (Figure 2-2) of the peach enzyme may not account for total EGase activity present in fruits. This EGase has maximum activity in 100 mM sodium citrate-phosphate buffer at pH 6.5, with very little activity observed below pH 5 and none at 4.0 (Hinton and Pressey, 1974). Optimal activity was observed at pH 6.0 in the presence of 10 mM Ca^{2+} (as CaCl_2) and more than 80 mM NaCl (Hinton and Pressey, 1974). EGase isoforms have been described in tomato fruit with pH optima at 5 and 7.0-7.5 (Pharr and Dickinson, 1973; Sobotka and Stelzig, 1974; MacLachlan and Brady, 1992) and the avocado EGase has optimal activity between pH 4.5 and 6.0 (Pesis et al., 1978). It is likely that fruit EGases are significantly active over the pH range 5-7.

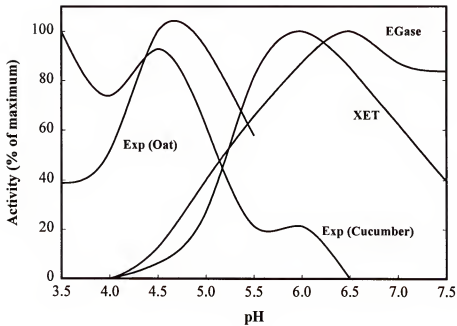


Figure 2-2. Effect of pH on the activity of enzymes capable of altering the cellulose-xyloglucan network. Data derived from Purugganan et al. (1997) (XET), Hinton and Pressey (1974) (EGase), McQueen-Mason et al. (1992) and Li et al. (1993) (expansin).

The Arabidopsis XET has optimal activity at pH between 6.0 and 6.5 and is unaffected by Ca^{2+} (10 mM) or K^{+} (100 mM) (Purugganan et al., 1997). Kiwifruit XET has optimal activity at pH 5.8 (Redgwell and Fry, 1993; Schröder et al., 1998). An oligosaccharide-activated xyloglucanase (xyloglucan depolymerase, possibly a buffer-insoluble XET) from tomato has considerable activity in the range 5-6 with maximum activity at pH 6 (Maclachlan and Brady, 1992).

Both pH and ionic strength affect the binding of XET to the cell wall (Redgwell and Fry, 1993; Schröder et al., 1998). Percy et al. (1996) solubilized 100% of XET

activity in kiwifruit with 0.4 M NaCl, but in apple about one quarter of the activity remained in the pellet even using 2 M NaCl at pH 6.4. Kiwifruit XET binds to the cell wall at pH 5.0, but not at pH 5.8, where the enzyme has optimal activity (Redgwell and Fry, 1993).

Expansins on the other hand, are active at pH values lower than 5, although the expansin from oat coleoptile retains 60% of its maximum activity at pH 5.5. The ripening-specific expansin (Rose et al., 1997) has not yet been biochemically characterized, but the high similarity in the amino acid sequence of all expansins so far identified hints at a common biochemical behavior.

Pectic Matrix

Pectins have several physiological roles. They constitute a network coextensive with the cellulose-xyloglucan framework, which has load-bearing ability (Shedletsky et al., 1990, 1992). The pectic matrix determines apoplastic porosity (Baron-Epel et al., 1988; Shedletsky et al., 1992), cohesion between adjacent cells (Knee, 1978), and transduces environmental signals (Aldington and Fry, 1993).

Pectic polymers are subjected to dramatic changes during fruit ripening. Modifications include deesterification, increased solubilization in water and chelators, deglycosylation, depolymerization and/or disaggregation. Solubilization, deesterification, deglycosylation and depolymerization are not distinct stages in the process of pectin degradation, but there is considerable overlap between these events.

Deesterification of Pectins during Ripening

The uronosyl residues in pectins are methylesterified during synthesis of the polysaccharide in the Golgi apparatus (Staehelin and Moore, 1995) and are thought to be deposited in the cell wall largely esterified. The degree of esterification decreases during ripening of tomato (Koch and Nevins, 1989), plum (Boothby, 1983), pear (Yoshioka et al., 1992), kiwifruit (Redgwell et al. 1992) and apricot (Femenia et al., 1998b). In strawberry, the degree of methylesterification remains unchanged during ripening (Wade, 1964). Similarly, in apple there is little or no change in overall methylesterification (Knee, 1978), but a closer analysis revealed that the degree of esterification decreases in the chelator-soluble pectins and increases in the water-soluble fraction (Yoshioka et al., 1992).

Deesterification may play critical roles in the regulation of cell wall metabolism. Highly methylesterified pectins can be depolymerized by lyase-mediated β -elimination, whereas PG-mediated hydrolysis requires demethylated carboxylic groups (Rexová-Benková and Markovic, 1976). PG has been studied extensively in association with ripening, and there is evidence that pectate lyase may also be present in strawberry fruit (Medina-Escobar et al., 1997). Desterification is also a mechanism for generating fixed negative charges in the apoplast, the implications of which will be discussed below.

Loss of Neutral Sugars during Ripening

The cell walls of most fleshy fruits exhibit a loss of non-cellulosic neutral sugars during ripening (Gross and Sams, 1984). The loss of neutral sugars has been evaluated by two types of observations: decreases in the neutral sugar content of cell walls prepared

from ripening fruit and increases in neutral sugars in soluble fractions of tissue homogenates. Neutral sugars can originate from hemicelluloses, pectins, and possibly from apoplastic proteins.

Neutral sugar composition varies among different fruit types (Gross and Sams, 1984; Redgwell et al., 1997). Galactose is the predominant neutral sugar in fruits of cucurbits, solanaceas and plum, arabinose in pome fruits, peach and nectarine, and xylose in berries and apricot. Fruits differ not only in neutral sugar composition but also in ripening-related changes. Although the levels of noncellulosic neutral sugars remain constant during ripening of plums and cucumbers, most fruit exhibit a net decrease in galactose and/or arabinose. Xylose levels increase slightly or remain constant during ripening of most fruit, but it is the major neutral sugar lost in apricot. In apple there is a significant loss in galactose and little decrease in arabinose (Bartley, 1976; Gross and Sams, 1984), whereas in pear the opposite trend is observed (Ahmed and Labavitch, 1980; Gross and Sams, 1984).

Galactose-rich polysaccharides represent primary substrates for deglycosylation during ripening. Cell wall galactosyl residues decline nearly 60 to 70% during the ripening of tomato and apple fruits (Wallner and Bloom, 1977; Gross and Sams, 1984; Redgwell et al., 1997). Galactose may play several roles during fruit ripening. The hydrolysis of galactosyl residues from pectins and hemicelluloses may contribute to fruit softening. Moreover, infiltration of galactose in tomato fruit increases ACC-synthase activity (Kim et al., 1987), a key regulatory enzyme in ethylene biosynthesis, and promotes the ripening-related biological activity of N-glycans (Yunovitz and Gross, 1994). In spite of the ability of galactose infiltrated into tomato fruit at high

concentrations (400 to 800 $\mu\text{g g}^{-1}$ f.w.) to promote ethylene synthesis (Kim et al., 1987), it does not appear to be responsible for the climacteric rise in ethylene production (Kim et al., 1991).

The loss of neutral sugars is an early event in cell wall disassembly during fruit ripening. The decline in galactose is initiated before the increase in polyuronide solubilization and continues during ripening (Bartley, 1974; Gross and Wallner, 1979). Deglycosylation of pectic and hemicellulosic polymers is a complex process, given the diversity in neutral sugar composition and glycosidic linkages involved. Loss of galactose can be accounted for by hydrolytic release or by cell wall turnover by incorporation of polymers with low galactose content (Pressey, 1983; Lackey et al., 1980). β -Galactosidase isozymes may participate in the removal of galactose residues (Pressey, 1983; De Veau et al., 1993). Loss of neutral sugars can also occur independently of glycosidase activity, as shown by the loss of arabinose in pear as a consequence of PG-mediated hydrolysis of arabinose-rich pectic polymers (Ahmed and Labavitch, 1980).

Solubilization of Pectic Polymers

Electron microscopy studies of ripening apple and pear (Ben-Arie and Kislev, 1979) and tomato (Crookes and Grierson, 1983) fruits show a progressive degradation of the middle lamella accompanied by swelling of the primary wall. The changes in the ultrastructure of the cell wall are particularly evident in the middle lamella, evidence of increased pectin solubilization, possibly promoted by depolymerization. The solubility of pectins is typically determined by sequential fractionation of cell wall material in aqueous

solvents. Water and buffers containing chelators and dilute alkali are often used. Water-soluble pectins are thought to be soluble in the apoplast, whereas chelator- and alkali-soluble pectins are thought to be ionically and covalently bound, respectively (Fry, 1988).

Pectin solubilization is altered during fruit ripening. In ripening tomato, water-soluble pectins increase during ripening, apparently at the expense of the Na_2CO_3 -soluble polymers, while CDTA-soluble pectins remain relatively constant (Carrington and Pressey, 1996).

Depolymerization of Pectic Polymers

The extent of pectin depolymerization during ripening varies among fruits, ranging from minimal depolymerization in strawberry (Huber, 1984), slight downshift in molecular mass in muskmelon (Ranwala et al., 1992), to extensive degradation in avocado (Huber and O'Donoghue, 1993). The mechanism of pectin solubilization and depolymerization and how these processes contribute to textural changes during fruit ripening is a controversial matter. Pectin solubilization occurs in the absence of depolymerization in ripening strawberry (Huber, 1984) and tomato locule tissue (Cheng and Huber, 1996), which contain no detectable endoPG activity. In persimmon fruit, however, with no detectable endoPG activity, both solubilization and depolymerization were found to occur (Cutillas-Iturrilade et al., 1993).

Enzymes that Alter the Pectic Matrix

Three enzymes have been implicated in the ripening-related changes in the pectic matrix: pectinmethylesterase, β -galactosidase, and polygalacturonase.

Pectinmethylesterases

Pectinmethylesterase (PME; EC 3.1.1.11) catalyses the deesterification of methylated pectins, releasing methanol. Besides its role in the cell wall metabolism, PME also affects other aspects of fruit ripening. The methanol generated by PME is a competitive inhibitor of alcohol dehydrogenase (EC 1.1.1.1) in ripening fruit and is believed to be responsible for the suppression in ethanol production during fruit ripening (Frenkel et al., 1998).

Deesterification must occur before PG can hydrolyze high methoxyl pectins (Jansen and MacDonnell, 1945; Jansen et al., 1945; Pressey and Avants, 1982; Seymour et al., 1987; Koch and Nevins, 1989). In agreement with these observations, molecular down-regulation of PME activity in tomato fruit resulted in decreased deesterification, and reduced depolymerization and solubility of pectins at the ripe stage (Tieman et al., 1992). Reduced PME activity, however, had little influence on ripening-related softening of whole fruit although it resulted in extensive loss of integrity in overripe fruit (Tieman and Handa, 1994).

In tomato pericarp, PME activity increases more than 50-fold during development through the mature-green stage, and then declines slightly during ripening (Gaffe et al., 1994). In contrast, Tucker et al. (1982) observed a 30% increase in PME activity during tomato ripening. Other authors have observed an increase in PME activity in tomato pericarp through the breaker/turning stage and a slight decline thereafter (Hobson, 1963; Pressey and Avants, 1982; Harriman et al., 1991). PME activity increases about 4 to 5-fold during ripening of papaya (Lazan et al., 1995), decreases in avocado (Zauberman

and Schiffmann-Nadel, 1972; Awad and Young, 1979), and remains constant in banana (Brady, 1976). Despite the trend in total extractable PME activity, it is clear that different isozymes are differentially regulated during ripening. Immature tomato fruit have a PME isozyme composition similar to vegetative tissues. Three fruit-specific isozymes become evident in 20-day-old fruit. One of these (pI 8.5) becomes the dominant isoform at the mature-green stage and remains throughout ripening (Gaffe et al., 1994).

β -Galactosidases

β -Galactosidases (*exo*- β [1 $\rightarrow$ 4]-D-galactopyranosidase; β -gal; EC 3.2.1.23) have been reported in many plants where they are present in buffer-soluble and high salt-extractable isozymes (Dey and Del Campillo, 1984). This review deals specifically with fruit cell wall β -gal, but it is noteworthy that β -gal show increased activity in other senescent organs, including daylily (*Hemerocallis* hybrid) petals (Panavas et al., 1998). This enzyme may be responsible for the decrease in cell wall galactose during carnation petal senescence (de Vetten and Huber, 1990). β -Gal catalyses the cleavage of β (1 $\rightarrow$ 4) galactosyl bonds. Endo-galactanase activity has not been detected in higher plants (Smith et al., 1998), but a role for such enzyme has been suggested based on the fact that galactose loss from cell walls is much higher than the potential for β -gal to release galactose in in vitro assays (Ross et al., 1993). β -Gals are also capable of transferase activity, transferring the galactosyl residue to an acceptor galactan, although the hydrolytic activity is higher than transglycosylation activity (Dey and Del Campillo, 1984; Bonnin et al., 1997). No information is available on whether transglycosylation activity exists in the cell walls of ripening fruit.

Total extractable β -gal activity has been reported to increase during ripening in tomato (Wallner and Walker, 1975; Carey et al., 1995; Sozzi et al., 1998), kiwifruit (Wegrzyn and MacRae, 1992; Bonghi et al., 1996) and muskmelon (Ranwala et al., 1992) fruits. In apple fruit, extractable β -gal activity has been reported to increase (Bartley, 1974) or remain constant during ripening (Ross et al., 1994). Tomato fruit contain three β -gal isozymes only one of which (β -gal II) is active against native galactans (Pressey, 1983). Pharr et al. (1976) found no β -gal activity associated with cell walls of tomato pericarp and reported that bulk tissue β -galactosidase activity did not change during ripening. Pressey (1983) showed that extractable β -galactosidase activity remained constant during ripening of tomato fruit but the proportion of the 3 isoforms is altered. The tomato β -gal II isoform is extracellular and ripening related (Smith et al., 1998; Sozzi et al., 1998). The activity of β -gal II reaches a maximum at the turning-pink stage (Carrington and Pressey, 1996), consistent with the maximum level of the transcript for this protein at the turning stage (Smith et al. 1998).

Polygalacturonases

Plants possess two enzymes capable of hydrolyzing the α -D-(1 $\rightarrow$ 4) glycosidic bond between adjacent galacturonic acid units: an exo- and an endo-acting polygalacturonase. In addition, pectin lyase (EC 4.2.2.2) cleaves the same bond by β -elimination. Pectin lyase enzyme is widespread among microorganisms (Rexová-Benková and Markovic, 1976), but its presence in plants has not been unequivocally documented. A cDNA from strawberry fruit with sequence similarity to pectin lyases hints at their possible presence (Medina-Escobar et al., 1997).

Exopolygalacturonase (ExoPG EC 3.2.1.67) catalyzes the stepwise removal of monomers from the non-reducing end of polyuronates (Pressey, 1987). ExoPG activity is highest in young immature tomato fruit, decreases to the mature-green stage and then increases 17% during ripening (Pressey, 1987). The enzyme has an apparent M_r of 47 kD, has optimal activity at pH 4.5 to 5.5 and is activated by Ca^{2+} (optimal at 0.42 mM), but not by Mg^{2+} (Pressey, 1987). Similarly, the peach and pear exoPG have optimal activity at pH 5.5, is activated by 0.4 mM Ca^{2+} (Pressey and Avants 1973, 1976). The optimum pH of the apple exoPG is 4.5-5.0, with half of the maximum activity at pH 4.2 and 5.6 (Bartley, 1978). Given the low levels of exoPG activity in ripening tomato, Pressey (1987) rejected the idea that exoPG plays an important role in pectin degradation. In fact, peach exoPG accumulates when the fruit are already very soft (Downs et al., 1992). It was suggested that exoPG may play a role in cell wall disassembly by cleaving the linkages between pectins and cell wall proteins (Pressey and Avants, 1976), but its role is likely to be related with the metabolism of signaling oligogalacturonates (García-Romera and Fry, 1995).

Polygalacturonase (endo- α -1,4-galacturonase; PG; EC 3.2.1.15) cleaves the $\alpha(1\rightarrow4)$ galacturonosyl linkages in the backbone of the pectic polymers. Early surveys revealed that PG activity was not ubiquitously distributed in fruits. Hobson (1962) detected PG activity in tomato, avocado, pear and pineapple, but not in persimmon, cucumber, tangerine, melon and grape fruits. Subsequent work has identified PG activity in cucumber (Saltveit and McFeeters, 1980) and the expression of a ripening-related gene encoding for PG in melon (Hadfield et al., 1998).

Tomato PG has been extensively reviewed (Giovannoni et al., 1992). Tomato fruit contain three glycosylated PG isozymes. PG1 has a Mr of about 115 kD pI of 8.6 and accumulates in the early ripening stages. PG2 A and B have Mr of 43 and 46 kD respectively, a pI of 9.4, and accumulate during the latter stages of ripening (Ali and Brady, 1982). Two endoPG isozymes (PG1 and PG3) have also been reported in banana (Pathak and Sanwal, 1998). PG3 accumulates later than PG1, and steadily increases during ripening, whereas PG1 activity starts before the climacteric rise, increases and then declines after the climacteric peak (Pathak and Sanwal, 1998).

Tomato PG mRNA is absent from immature fruit, starts accumulating at the onset of ripening and continues accumulating until the ripe stage (Grierson et al., 1985; DellaPenna et al., 1986). The beginning of the rise in climacteric ethylene production in tomato fruit precedes PG synthesis by about 20 h (Grierson and Tucker, 1983) and PG gene expression responds to low levels of ethylene (Sitrit and Bennett, 1998).

Other enzymes that may target the pectic matrix

The enzymes thus far discussed can target only a few of the glycosidic linkages present in the cell wall, namely 1→4 linked β-D-glucopyranosyl residues (XET and EGase), 1→4 linked α-D-galacturonosyl (exo- and endoPG), and 1→2 linked β-D-galactopyranosyl (β-gal). Cell wall polysaccharides contain many more glycosidic linkages, which are potential targets of enzyme activity.

α-Galactosidase activity (EC 3.2.1.22) has been identified in isolated tomato fruit cell walls (Pharr et al., 1976) and shown to increase during ripening (Sozzi et al., 1996). α-Galactosidase has also been identified in other fruits, including muskmelon (Ranwala

et al., 1992) and mango (Ali et al., 1995). Two isoforms of putative cell wall α -galactosidase have been reported in orange juice vesicles, only one of which is active against cell wall preparations but fails to release reducing groups from water soluble pectins (Burns, 1990), possibly due to few susceptible linkages in water-soluble pectins. Possible targets for this enzyme in the cell wall are RGII and structural proteins, which contain α -D-galactopyranosyl residues (Bacic et al., 1988).

β -1,3-Glucanase activity (EC 3.2.1.39) was extracted from tomato fruit at all ripening stages and a corresponding 12 kD enzyme has been identified (Wallner and Walker, 1975). α -Mannosidase activity (EC 3.2.1.40) increases during ripening of mango and bell pepper (Ali et al., 1995; Sethu et al., 1996), but in muskmelon very little α -mannosidase activity was found in salt released fractions (Ranwala et al., 1992). Xylanase activity remains constant during mango ripening (Ali et al., 1995) and decreases in ripening bell pepper (Sethu et al., 1996). α -Arabinosidase remains constant during ripening of mango (Ali et al., 1995) and a β -arabinosidase (EC 3.2.1.56) was identified in tomato locule tissue (Cheng and Huber, 1997). A putative target for α -arabinosidase could be the 1 $\rightarrow$ 2 linked α -L-arabinofuranosyl residues present in xyloglucans or the (1 $\rightarrow$ 5)- α -L- arabinofuranosyl in pectins, whereas extensin glycans β -arabinofuranosyl residues could be the substrate for β -arabinosidase.

Endo- β -mannanase (EC 3.2.1.78) activity accumulates at the mature-green stage and increases during tomato fruit ripening (Sozzi et al., 1996). Pressey (1989) purified an endo- β -mannanase from tomato fruit, capable of hydrolyzing β -1,4-mannosyl linkages. The activity is very low or absent in mature-green fruit, increases abruptly at the turning

stage, and continues accumulating during ripening. The enzyme has optimal activity at pH 4.5 to 5, Mr of 43 kD and pI of 9.3 as determined by isoelectric focusing.

These glycosidases are usually tested using artificial substrates or non-native polysaccharides, so it is difficult to assess their role in cell wall disassembly.

Regulation of Pectic Matrix Disassembly in Ripening Fruit

Transcriptional Regulation

Ethylene is a key regulatory hormone for expression of cell wall hydrolases in several stages of plant development. Ethylene regulates the expression of the pectin hydrolases, PG (Sitrit and Bennett, 1998) and β -galactanase (Sozzi et al., 1998) but apparently not PME. In nonclimacteric fruits, where autocatalytic ethylene production is absent, gene expression is regulated by other developmental factors.

Figure 2-3 summarizes the expression patterns of the major pectin modifying enzymes present in ripening tomato: the major PME isozyme (Harriman et al., 1991), β -gal II (Smith et al., 1998) and endoPG (DellaPenna et al., 1986).

In tomato fruit, the levels of PME transcript decrease sharply at the onset of ripening and remain at about 20 - 40% of their mature-green levels after the turning stage (Harriman et al., 1991). Transcripts for both β -galactanase (Smith et al. 1998) and PG (DellaPenna et al., 1986) start accumulating after the mature-green stage, with β -galactanase reaching a peak at the turning stage whereas PG accumulation continues into the ripe and overripe stages. The expression patterns suggest that these enzymes may act sequentially during ripening.

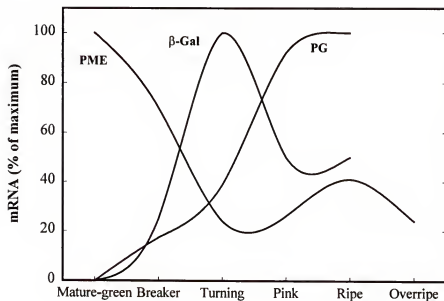


Figure 2-3. Expression of the major enzymes impacting the pectic matrix during fruit ripening. Data derived from Harriman et al. (1991) (PME), Smith et al. (1998) (β -gal) and DellaPenna et al. (1986) (PG).

Regulation of Pectin Disassembly by pH and Minerals

Figure 2-4 shows the effect of pH on the activity of PME, β -gal and PG. The data for tomato PME isozymes were derived from Warrilow and Jones (1995) and is in agreement with the results obtained by Giovane et al. (1994). The data for β -galactanase were obtained from the kiwifruit enzyme (Ross et al., 1993). The pH-activity profile of kiwifruit β -gal is agreement with the information provided by Pressey (1983) for the tomato β -gal II. The effect of pH on tomato PG1 is as determined in 50 mM sodium

acetate (Pressey and Avants, 1973) and for PG2 assayed in 25 mM KCl, 20 mM potassium acetate (Chun and Huber, 1998).

Taken together, the activity of PME, PG and β -gal spans the entire range of pH likely to occur in cell walls (Figure 2-4). At pH 6, only PME A and B exhibit a substantial fraction of their maximum activity (Warrilow and Jones, 1995). The pH optima in the alkaline region determined for PME should be taken with caution due to the confounding effects of alkaline deesterification. Even subtracting chemical deesterification from total deesterification in reactions containing PME may not reflect the precise nature of the reaction, since PME activity is higher against partially deesterified pectins (Rexová-Benková and Markovic, 1976). The activity of PG2 at low ionic strength and in the presence of K^+ has a maximum at pH 5, whereas PG1 is more active at slightly lower pH (Pressey and Avants, 1973; Chun and Huber, 1998). β -Gal has optimal activity in the pH range 4.5-5.0 and retains more than 50% of its activity from 4.0 to 5.5 (Ross et al., 1993). PG activity overlaps with β -gal and, from the three PME isozymes, only PME A (Warrilow and Jones, 1995) retains 9 to 78% of its maximum activity at pH values ranging from 4 to 5.5, respectively.

Although there is agreement between different studies about the optimal pH and mineral composition of buffers for in vitro activity, there are many examples of interactions between pH, mineral composition, ionic strength and nature of the substrate. It seems thus appropriate to review some of the results that highlight these interactions.

PME from fruits show relatively high pH optima. The 3 PME isozymes from tomato fruit have maximum activity over the pH range from pH 7.5 to 10 and only PME A still exhibits substantial activity (60 % of maximum) at pH 5 (Tucker et al., 1982;

Pressey and Woods, 1992; Giovane et al., 1994; Warrilow and Jones, 1995). When assayed at low ionic strength, the pH optima for tomato fruit PME shifted to higher values and activity occurs over a narrower pH range (Pressey and Avants, 1972, Brady, 1976, Pressey and Woods, 1992). PME isozymes 3 and 4 from tomato fruit assayed in the presence of high ionic strength (as NaCl), 250 mM and 100 mM respectively, exhibit substantial activity at pH values as low as 3.0 (Pressey and Woods, 1992). The in vivo relevance of this high ionic strength response is questionable, however, since the ionic strength of the apoplast is likely much lower than the assay levels (discussed below).

Cations greatly stimulate the activity of plant PMEs (Rexová-Benková and Markovic, 1976). Ca^{2+} at 5 mM is required for maximum tomato PME (possibly the isozyme that Pressey and Avants (1972) described as PME 4) activity, but higher levels are inhibitory (Lee and Macmillan, 1968). K^+ stimulates the activity of both lemon peel and jelly fig (*Ficus awkeotsang*) PME, but affects the pH-response of each enzyme in a different way (Komae et al., 1989). In the presence of 65 mM K^+ , the pH optimum of jelly fig PME shifts from 5.4 to 8, whereas the pH optimum for lemon PME (7.5-8) is unaffected by K^+ levels. *Ficus awkeotsang* PME has optimal activity in the presence of 12.5 to 24.5 mM Ca^{2+} in the pH range from 5.4 to 7 (Komae et al., 1989).

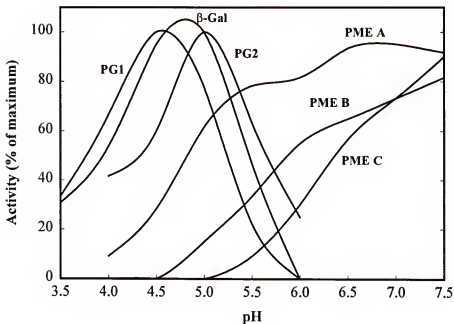


Figure 2-4. Effect of pH on the activity of pectin modifying enzymes in ripening fruit. Data derived from Warrilow and Jones (1995) (PME), Ross et al. (1993) (β -gal), Pressey and Avants (1973) (PG1) and Chun and Huber (1998) (PG2).

The activity of fruit β -gals (assayed against *p*-nitrophenyl- β -D-galactopyranoside) is optimal in the pH range 2.8 to 4.2 (Burns, 1990; Ross et al., 1993; Andrews and Li, 1994; Ross et al., 1994; Ali et al., 1995; Carey et al., 1995; Biles et al., 1997). Relatively few studies have addressed the effect of pH on β -gal activity against native, cell wall galactans. Nonetheless, data from kiwifruit β -gal suggest that the pH optimum of β -gal toward galactans is higher than that against *p*-nitrophenyl- β -D-galactopyranoside, and the activity range is narrower (Ross et al., 1993). Against *p*-nitrophenyl- β -D-galactopyranoside, the enzyme is active at pH values from 2 to 8 with an optimum at 3.2. When galactan is used as a substrate, the enzyme has a narrower activity range and a

higher pH optimum. Activity is optimal at pH 4.9 and is completely inhibited at pH 6.0. Similarly, tomato β -gal II has a pH optimum of 3.5 against *p*-nitrophenyl- β -D-galactopyranoside, and 4.5 against a spruce galactan (Carey et al., 1995). Pressey (1983), however, reported identical pH optima (4.2) for the activity of tomato β -gal II against *p*-nitrophenyl- β -D-galactopyranoside and galactan. Bartley (1974) found that apple β -gal exhibited a bimodal pH response, with optima at 4.0 and 8.0.

There are conflicting data on the effects of inorganic minerals on β -gal activity (against *p*-nitrophenyl- β -galactoside). The activity of Japanese pear cell wall-bound β -gal is not affected by 2 mM Ca^{2+} or Mg^{2+} or EDTA (Kitagawa et al., 1995). Similarly, NaCl, KCl and CaCl_2 (0.15 - 0.75 M) had little effect on the activity of mango β -gal isozymes (Ali et al., 1995). On the other hand, 1 mM Mg^{2+} doubled the activity of sweet cherry β -gal in 40 mM Na-acetate, pH 5.0 (Andrews and Li, 1994).

The optimum pH for PG is influenced by ionic strength, and the degree of polymerization (DP) and esterification of the substrate. Maximum PG-mediated solubilization of pectins from isolated cell walls occurs at pH 5 (for tomato PG1 and PG2) in PME deesterified cell walls, but at pH 3.3 and 3.7 for PG1 and PG2 respectively, for native cell walls (Pressey and Avants, 1982). The activity of tomato PG against pectins with a DP of 3 or 4 has pH optima of 3.5 and 4.5 but against pectins with a DP of 14 the pH optima are 2.5 and 4.5 (Patel and Phaff, 1960). Consistent with these results, autolysis of ripe tomato cell walls is higher at pH 2.5 and 4.5 than at 6.5 and higher (Rushing and Huber, 1987). Peach PG2 has an optimum pH at 4.5 with large substrates, but the pH optimum shifts to lower values as the substrate DP decreases (Pressey and Avants, 1973).

The role of individual minerals is illustrated by the following examples. In buffers with the same ionic strength and equimolar concentrations, the activity of avocado PG is higher in the presence of Na^+ than K^+ or phosphate (Reymond and Phaff, 1965). Tomato PG activity is inhibited by Ca^{2+} , probably by the interaction of the cation with the substrate (McColloch and Kertesz, 1948; Buescher and Hobson, 1982). The optimum pH for tomato PG2 activity against polygalacturonic acid in the presence of 120 mM K^+ is 1.5 units higher than in the presence of 170 mM Na^+ (Chun and Huber, 1998). The difference is only 0.5 units when the substrate is cell wall material (Chun and Huber, 1998), possibly due to differential ion-exchange properties of the substrates. Although there may be some confoundment of the effects of ion species and ionic strength the data suggest that, when compared to Na^+ , K^+ inhibits PG activity at pH values between 3 and 4.5 but stimulates at higher pH values. Increasing $[\text{K}^+]$ from 5 to 100 mM results in increased PG-mediated pectin solubilization at pH equal or lower than 5, but not at higher pH (Chun and Huber, 1998).

Apoplastic pH and Mineral Composition

From the data on the transcriptional regulation presented above, it is clear that the enzymes have different patterns of accumulation during ripening. From the pH-activity profiles of the various cell wall enzymes, it is also clear that the *in vivo* activity of the enzymes is differentially regulated by pH, individual minerals and ionic strength encountered in the cell wall domains where the substrate resides.

Apoplastic pH, mineral composition, and ionic strength could provide a means to integrate the activity of different enzymes to achieve the concerted disassembly of the

cell wall. This section summarizes the data on apoplastic pH and mineral composition in ripening fruit, introduces the concept of apoplastic ionic domains and discusses several ways by which pH, mineral composition, and ionic strength might regulate cell wall disassembly.

Apoplastic pH in Ripening Fruit

Reported values for apoplastic pH of vegetative tissues range from 4 to 7 and depend on the species, tissue and metabolic activity (Grignon and Sentenac, 1991). The role of pH on cell wall metabolism in vegetative organs has been widely recognized and incorporated in the acid-growth theory (Rayle and Cleland, 1992). The few studies that addressed the apoplastic pH of ripening fruit have reported values that generally fall within the range mentioned above. Using microelectrode measurements, Ballarin-Denti and Antoniotti (1991) found an apoplastic pH of 7.8 for squash fruit, although the authors gave no indication of the maturity. Ruan et al. (1995) extracted apoplastic fluid from immature tomato fruit and reported a pH of 6.0. In one report the apoplastic pH was monitored during ripening of peach and apricot by equilibrating sections of the mesocarp in water. Early in ripening the apoplast of the mesocarp has pH values near 7.0. During ripening, apoplastic pH drops to 4.3 in peach and 3.4 in apricot (Ugalde et al., 1988). Together, these reports provide evidence that the apoplastic pH of pre-ripe fruit approaches neutrality, and acidifies as much as 3 pH units during ripening. Nevertheless, the dynamics of apoplastic pH during ripening is very poorly documented.

Apoplastic Mineral Composition

Apoplastic minerals play several roles on cell wall metabolism. Some, like boron and calcium have structural roles in the formation of RGII dimers (Ishii et al., 1999) and polyuronide aggregates (Grant et al., 1973; Thakur et al., 1997), respectively. Minerals also affect the binding and catalysis of cell wall enzymes, by effects on substrate or protein conformation. Ionic interactions are strongly reduced by increasing the ionic strength of the solution.

Apoplastic mineral profiles have been well characterized in vegetative tissues in relation to mineral nutrition, stomata function, nastic movements, and elongation growth (Grignon and Sentenac, 1991); however, the mineral composition of the apoplast in fruit tissues and its dynamics during ripening remain largely unknown.

The mineral composition of a pressure-extracted apoplastic solution from immature tomato pericarp was determined by Ruan et al. (1996). The apoplastic solution is more dilute than the bulk fluid, with an osmolality about one-third of the bulk fluid. Inorganic ions account for about 45% of the total apoplastic solutes. Potassium is the major apoplastic mineral, (20 mM), followed by chloride (9 mM), calcium (6-7 mM), magnesium (5 mM), phosphorus (3 mM), sodium (0.5 mM) and ammonium (0.5 mM).

The dynamics of the change in mineral composition during fruit ripening remain elusive. Tissues of ripening fruits leak increasing amounts of potassium and to a lesser extent magnesium into isotonic bathing solutions (Ferguson and Watkins, 1981), whereas leakage of calcium remains constant or decreases (Ferguson and Watkins, 1981; Dawson et al., 1993), probably due to increased cation exchange capacity of the cell wall. These experiments suggest that the apoplastic mineral environment is modified during ripening.

Using a nonaqueous fractionation method, MacDougall et al. (1995) attempted to estimate the levels of apoplastic calcium, potassium, magnesium and organic acids, of the pericarp of a wild tomato species (*Lycopersicon pimpinellifolium*). They divided the level of minerals and acids in the samples by the uronic acid content and regressed that value against an independent variable obtained by dividing phosphate levels by the uronic acid content. Phosphate was used as an indicator of cytoplasmic contamination and the estimated value for apoplastic minerals/acids was obtained by extrapolation to a value of zero (i. e. no phosphate) for the independent variable. The appropriateness of this methodology is questionable, however, since the two variables (mol mineral/mol uronic acid and mol phosphate/mol uronic acid) are correlated and not independent. In fact, two independently generated series of random numbers do not correlate with each other, but if both are divided by the same series of random numbers a good linear correlation will arise. This is a property of numbers, with no relation to biological or chemical effects. Moreover, the apoplast of immature tomato reportedly contains phosphate (Ruan et al., 1996), whose levels might change during ripening. The levels of apoplastic Ca^{2+} and Mg^{2+} in unripe fruit were estimated at 60 and 18 nmol mg^{-1} d. w. respectively, and remained unchanged during ripening. MacDougall et al. (1995) determined the percentage of Ca^{2+} and Mg^{2+} that is retained by the pellets resulting from centrifugation (5000g) of an homogenate of a wild tomato (*L. pimpinellifolium*) pericarp in distilled water. Compared with unripe fruit, the pellet of ripe fruit retained 3 to 8% more Ca^{2+} and 13 to 37% more Mg^{2+} . The fact that pellet retention decreased when the homogenates were stirred for 1 h prior to centrifugation indicates that the values obtained by this procedure are influenced by factors other than the in vivo partitioning between soluble

and pellet-bound forms. One factor involved is probably the increased chelation of divalent cations by organic acids. Estimated levels of apoplastic K^+ increased during ripening from 10 to 15% of total K^+ in the tissue.

Calcium affects cell wall integrity in ripening fruit (Poovaiah et al., 1988). It has a structural role via complexing with polyuronates, as postulated by the egg-box model (Grant et al., 1973), and a regulatory role, as the apoplast functions as a reservoir for Ca^{2+} acting as a secondary messenger in signal transduction pathways.

The quantification of apoplastic Ca^{2+} has proven difficult. Traditional approaches involving the partitioning of Ca^{2+} between soluble and bound forms using a variety of solvents do not accurately reflect specific forms of Ca^{2+} (Ferguson et al., 1980). Even so, much of the literature on apoplastic calcium has relied on these methodologies. Bound Ca^{2+} in tomato pericarp decreases at the onset of ripening, with levels remaining relatively constant with further ripening (Suwwan and Poovaiah, 1978; Rigney and Wills, 1981). Brady et al. (1985), however, found no changes in calcium partitioning (bound vs. soluble) during ripening. Imaging techniques have also been applied to the analysis of apoplastic calcium levels in ripening fruit. Energy dispersive X-ray analysis of apoplastic calcium revealed that apoplastic calcium increases during ripening of tomato pericarp, but not in the placental tissue or in peach mesocarp (Burns and Pressey, 1987). Secondary ion mass spectrometry and electron-energy-loss spectroscopy have revealed that calcium is not uniformly distributed in the apoplast, but accumulates predominately near intercellular spaces (Roy et al., 1995; Huxham et al., 1999), where pectins are more deesterified (Knox et al., 1990).

The apoplastic pH and mineral composition of ripening fruit have been the object of relatively few studies. Accordingly, an assessment of the potential role of pH and minerals in the modulation of enzyme activity *in vivo* is largely speculative. The remainder of this section discusses factors that are likely to be involved in the control of apoplastic ionic properties and their effect on cell wall disassembly.

Apoplastic Ionic Domains

Plant cell walls are not homogeneous but, rather, are composed of domains that differ in polysaccharide composition (Lynch and Staehelin, 1992) and pH (Guern et al., 1991). The spatial distribution of plasma membrane ion channels and ATPases, and the density and distribution of fixed negative charges can generate localized ionic domains. pH gradients as large as 3 units have been documented within cell walls (Grignon and Sentenac, 1991) and significant gradients are likely to exist for other ionic species. The evidence for these domains is largely indirect. Although they remain poorly characterized, the presence and properties of apoplastic domains are likely to play important roles in the regulation of cell wall metabolism.

Of the factors addressed above that might contribute to the generation of apoplastic ionic domains, the distribution and density of polyanionic pectins is the one that is best documented in ripening fruit. The distribution of methylesterified residues varies among organs, tissues, and within individual cell walls (Knox et al., 1990; Lynch and Staehelin, 1992; Femenia et al., 1998a). The degree of pectin methylesterification declines during ripening of many, but not all fruit (see section on Pectin Deesterification during Ripening). However, the progression of deesterification during ripening is

regulated. Using immunocytological techniques, Roy et al. (1992) showed that in cherry tomato fruit, most of the partially unesterified pectins are localized in the middle lamella at the mature-green stage. During ripening, deesterification increases toward the plasma membrane and extends throughout the primary wall in fully ripe fruit (Roy et al., 1992).

Deesterification may generate fixed negative charges depending on the pKa of the cell wall material. Cell walls (*Lupinus luteus* and *Vicia faba*) have a pKa near 5.0 whereas the pK of purified pectin and galacturonic acid is 3.2 (Sentenac and Grignon, 1981). For a given pH and pK, the Henderson-Hasselbach equation allows the calculation of the proportion of negative charges from unprotonated carboxyl groups. Assuming that the cell wall has a pH of 6.5 and pK 5.0, the concentration of the anionic species is more than 30 times higher than the protonated form. At pH 4.5, the protonated form is 3 times more abundant than the anionic species. Assuming that pectic microdomains within the cell wall have a pK of 3.2, the ratio of anionic/protonated carboxyl groups would be 2000 at pH 6.5 and 20 at pH 4.5. In any case, the number of negative charges in the pectins would vary 100-fold over the pH range from 4.5 to 6.5.

The density of negative charges in the apoplastic domain will influence its ionic environment. Models for electrostatic interactions describe ions gradients between the charged surface and the bulk solution as an exponential function of ψ/T , where ψ is the electrical potential of the wall and T the absolute temperature (Hartley and Roe, 1940; McLaughlin, 1989). These considerations show that, at a given temperature and in the absence of metabolic activity, the cell wall electrical potential is the sole determinant of the local concentration of mobile cations, including protons. Moreover, the apoplastic pH has a profound effect on the electrical potential, by affecting the dissociation of the

pectic carboxyl groups. The electrical potential of the apoplast affects also the activity of several voltage-regulated ion channels, which together with lack of homogeneity of the apoplastic solution will contribute to the formation of ionic domains.

Although arguably predominant, pectins are not the only source of fixed electrical charge in the apoplast. Cell wall proteins span a wide range of pI values. Extensins have pIs around 11, whereas arabinogalactan proteins (AGP), have a pI near 1.5 (Fry, 1988). Thus extensins can contribute positive charges, whereas AGP and pectins contribute to the net negative charge in the cell wall. These proteins may be the major determinants of electrical potential in specific apoplastic domains.

Immobilized Enzymes

Many cell wall enzymes have restricted mobility in the apoplast. Cell wall enzymes have pIs ranging from 5 for some β -gals (De Veau et al., 1993) and EGases (Awad and Lewis, 1980; Kanellis and Kalaitzis, 1992) to about 9 for PME (Tucker et al. 1982, Warrilow et al. 1994). Enzymes with basic pIs, including XET, PME and PG are likely to be immobilized in the cell wall whereas enzymes with acidic pI (β -gal) can be electrostatically bound to extensins in the pH range of the apoplast. Apoplastic enzymes can also be physically trapped in the cell wall (Baron-Epel et al., 1988).

The immobilization of an enzyme on a charged surface dramatically alters its kinetic properties and pH response (Katchalski et al., 1971; Ricard et al., 1981). Acid phosphatase exhibits classic Michaelis-Menten kinetics in free solution, whereas in the native cell wall it exhibits negative co-operativity, likely due to the repulsion of the substrate by the polyanionic wall matrix (Crasnier et al., 1985). This co-operativity is

alleviated in the presence of high ionic strength buffer or high calcium levels (Crasnier et al., 1985).

The catalytic behavior of cell wall enzymes is likely to be different in the apoplast, where the enzyme mobility is restricted, than in free solution. Rushing and Huber (1990) showed that PG binds tightly to cell walls and has restricted mobility. PG activity provides interesting examples of the interactions between pH and the charge density of the substrate. Peach PG has an optimum pH at 4.5 with large substrates, but the optimum shifts to lower values with lower DP substrates (Pressey and Avants, 1973). Similar effects of substrate DP were described for yeast PG (Demain and Phaff, 1954; Luh and Phaff, 1954). These differences in pH optima could be accounted for by the fact that high molecular mass pectins are able to induce lower pH in the microenvironment where enzyme binding and catalysis occur.

A Role for Apoplastic pH and Mineral Composition in Regulating Cell Wall Disassembly

There are a number of mechanisms by which the apoplastic environment might regulate cell wall disassembly in ripening fruit. One involves a temporal, ripening-related change in apoplastic pH and mineral composition. A decline in apoplastic pH from about 7 to 4.5 or lower, as suggested by the unconfirmed report of Ugalde et al. (1988) for peach and apricot fruits, covers a wide range of enzymic activities and could facilitate the sequential action of enzymes that target the cellulose-xyloglucan and pectic networks (Figures 2-2 and 2-4). Another possibility relates to the apoplastic ionic domains. Spatial heterogeneity in pH and mineral distribution could provide initiation points for catalytic activity, such that different enzymes are simultaneously active within different ionic

domains. The temporal regulation could be coexistent with this spatial, domain-dependent regulation as a result of changing electrical properties due to the activity of cell wall enzymes or membrane transporters. Enzymic degradation of pectic matrix results in increased porosity of the cell wall and facilitated diffusion of large proteins (Baron-Epel et al. 1988). This could facilitate the intermixing of the apoplastic solution and the movement of enzymes between domains as ripening progresses. A third putative mechanism for the regulation of cell wall disassembly is nonenzymic. Fishman et al. (1989) have shown, through a combination of size-exclusion chromatography and curve fitting techniques, that chelator and alkali-soluble pectins from tomato pericarp are composed of five distinct macromolecular species, with relative molecular sizes arranged in the geometric progression 1:2:4:8:16. In the presence of 50 mM NaCl, the larger species partially dissociated, generating smaller species, indicating that some portion of cell wall pectins are held together by noncovalent interactions. Changing mineral levels in the apoplast of ripening fruit may provide such mechanism.

Cell wall enzymes are transcriptionally regulated during fruit ripening. Cell wall hydrolases have also different pH-activity responses and are modulated by minerals. Moreover, the apoplast of ripening fruit contains domains that are likely to provide different ionic conditions. Apoplastic pH and mineral composition could provide an additional means for regulating the disassembly of fruit cell walls, by the sequential action of cell wall enzymes or their spatial distribution in ionic domains.

CHAPTER 3

PERICARP SOFTENING, PECTIN SOLUBILIZATION AND MOLECULAR MASS IN RIPENING AND CHILLED TOMATO FRUIT

Introduction

Ripening of many fruits involves disassembly of the cell wall structure, which is considered a main determinant of fruit softening. Although a number of cell wall polysaccharides are modified during fruit ripening, pectins are particularly impacted. Solubilization and depolymerization of polyuronides occur during the ripening of many fruits (Huber, 1983a). Pericarp of ripening tomato fruit shows a progressive dissolution of the middle lamella (Crookes and Grierson 1983). Polygalacturonase (PG; EC 3.2.1.15) is involved in pectin depolymerization, but its role in solubilization is less clear. Whereas water-soluble pectins were reduced in PG-deficient tomato fruit (Carrington et al., 1993), chelator-soluble pectin levels were similar in PG-antisense and wild-type fruit (Smith et al., 1990).

Tomato fruit develop chilling injury when stored for periods at low temperature (below 9-12 °C, depending on the developmental stage) and subsequently transferred to higher temperature (Hobson, 1987; Efiuvwevwere and Thorne, 1988). Storage at low temperature reduces whole fruit (Hobson, 1987) and pericarp firmness (Jackman et al., 1990; Marangoni et al., 1995) of mature-green/breaker tomatoes, even though PG does

not accumulate in mature-green fruit stored under chilling conditions (Jackman et al., 1992; Marangoni et al., 1995). Changes in pectins during chilling injury have received limited attention, although ultrastructural evidence showed that cell walls are affected by chilling, especially the pectin-rich middle lamella (Marangoni et al., 1989).

The following experiments were conducted to study fruit softening and pectin disassembly in chilled and ripening non-chilled fruit. PG accumulation is inhibited in mature-green tomatoes stored at low temperature, but resumes after transfer of chilled fruit to higher temperature, paralleling fruit softening (Jackman et al., 1992). Transgenic tomato fruit with down-regulated PG were used to determine the role of PG in chilling-induced pectin metabolism.

Material and Methods

Plant material

Tomato (*Lycopersicon esculentum* Mill.) fruit from line 1436 (Calgene, Davis, CA) containing a PG-antisense construct and untransformed (wild-type) fruit were used in the experiments. The plants were field-grown in Gainesville, Florida, in spring 1997. Fruit were harvested at the mature-green stage, washed in 2 mM sodium hypochlorite for 2 min, rinsed with water, and air-dried before storage at the indicated temperatures.

Storage temperatures

Fruit were stored at 5 or 12 °C for 21 days, after which time the fruit were transferred to 18 °C for 5 days. During storage, fruit were kept in 19 L plastic buckets aerated with water-saturated air at a flow rate of approximately 20 mL min⁻¹ kg⁻¹ during

storage at 5 °C and 75 mL min⁻¹ kg⁻¹ for storage at 12 and 18 °C. Flow rates were calculated based on the respiration rates for mature-green tomatoes provided by Hardenburg et al. (1986) to prevent increase in temperature and excessive accumulation of CO₂.

Firmness and color determination

Pericarp firmness was determined using disks excised from the equatorial region of the fruit with a cork borer (15.5 mm diameter). The epidermis and endodermis tissues were removed with two surgical blades mounted in parallel on a frame, yielding disks of 3 mm thickness. The disks were compressed with a Chatillon firmness tester (model HTMC, John Chatillon & Sons, New York, NY) equipped with a 12 mm diameter flat probe. The probe was driven at a speed of 1.4 cm min⁻¹ and the force required to deform each disk 2.5 mm was recorded. Five disks were measured from each of three fruit sampled immediately before storage, after 21 days at 5 or 12 °C, and after 5 days at 18 °C following storage at 5 or 12 °C.

Color measurements (CIELAB system) were determined at the equatorial region of each fruit using a Minolta CR-200 reflectance colorimeter (Ramsey, NJ) with a D₆₅ illuminant. The colorimeter was calibrated with a white standard tile. The results are presented as lightness (L), chroma (C*) and hue angle (h°). The chroma and hue were calculated from the measured a* and b* values using the formulas $C^* = (a^{*2} + b^{*2})^{1/2}$ and $h^{\circ} = \arctan(b^*/a^*)$ (McGuire, 1992).

Preparation of ethanol insoluble solids

Ethanol insoluble solids (EIS) were prepared by homogenizing 20 g of frozen pericarp tissue in 80 mL of 95% ethanol. The homogenates were kept at -20°C overnight, and then centrifuged at $8000g$ for 10 min at 4°C in a Beckman J2-21 centrifuge (JA-20 rotor, Fullerton, CA). The pellet was suspended in Tris-buffered phenol, pH 7.0 for 60 min to inactivate enzymes (Huber, 1991). The phenol suspension was adjusted to 80% ethanol, maintained overnight at -20°C to precipitate structural polysaccharides, then filtered through Whatman (Springfield, Kent, UK) GF/C glass fiber filters. The residue (EIS) was suspended in 25 mL chloroform:methanol (1:1) for 30 min, filtered through a GF/C filter, and washed with 100 mL of acetone. EIS were vacuum dried, incubated overnight in an oven at 34°C , and stored in a dessicator at room temperature.

Extraction and chromatography of EIS pectic polysaccharides

Total uronic acids in EIS were determined by the method of Ahmed and Labavitch (1977). Water- and CDTA-soluble pectins were extracted by suspending 20 mg of EIS in 7 mL of distilled water or 50 mM sodium acetate, 50 mM mM CDTA, pH 6.5, respectively. After 6 h at room temperature, the suspensions were filtered and uronic acid in the filtrates determined by the *m*-phenylphenol method (Blumenkrantz and Asboe-Hansen, 1973).

Solubilized pectins were separated by size using a Sepharose CL-2B column (29 cm long x 1.5 cm diameter) operated in 200 mM ammonia acetate, pH 5.0 as the mobile phase (Mort et al., 1991). The column was loaded with 500 μg of uronic acid equivalents

in 1 mL and fractions of 2 mL were collected. Uronic acids were determined for each fraction by the *m*-phenylphenol method and expressed as a percentage of total uronic acids recovered.

Results

Color and pericarp softening in ripening and chilling-injured fruit

Since surface color measurements were similar for PG-antisense and wild type fruit at each sampling date, values are presented averaged over both lines. Tomato fruit stored at 5 °C for 21 days showed a slight decrease in hue (increased yellowing), associated with an increase in lightness (L^*) (Table 3-1) compared with prestorage fruit, but did not develop red hue, indicating that ripening was arrested during storage at 5 °C. Fruit stored at 12 °C for 21 days had become red, exhibiting a marked decrease in hue (Table 3-1). The hue of fruit transferred from 12 to 18 °C remained unchanged for 5 days, whereas that of fruit stored at 5 °C for 21 days continued to decrease upon transfer to 18 °C.

Pericarp firmness decreased by 5% in fruit stored for 21 days at 5 °C, whereas pericarp firmness of fruit stored at 12 °C had declined by 25% and 40% for PG-antisense and wild type, respectively (Figure 3-1). Upon transfer of fruit stored at 5 or 12 °C to 18 °C, softening accelerated although the rate of softening was similar in all treatments. Reduced PG had no effect on the firmness of pericarp tissue from fruit stored at 5 °C but PG-antisense fruit at 12 °C remained firmer than the wild-type throughout storage and ripening.

Table 3-1. Surface color of tomato fruit during storage at 5 or 12 °C. Fruit were stored at 5 or 12 °C for 21 days and then transferred to 18 °C for 5 days. Values followed by the same letter (by column) are not significantly different at 5% level by the Duncan's multiple range test.

Days	L*		Chroma		Hue angle	
	12 °C	5 °C	12 °C	5 °C	12 °C	5 °C
0	59.7 a	59.7 b	38.2 a	38.2 a	117.4 a	117.4 a
21	50.1 b	63.9 a	40.1 a	37.7 a	58.6 b	111.9 b
	18 °C		18 °C		18 °C	
5	49.2 b	65.4 a	41.4 a	35.9 a	54.9 b	98.3 c

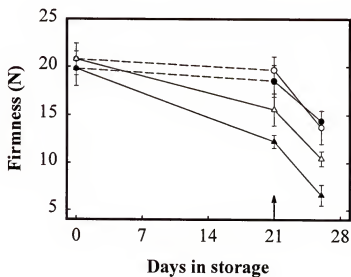


Figure 3-1. Pericarp firmness of PG-antisense (Δ , $\circ$) and wild-type ($\blacktriangle$, $\bullet$) tomato fruit stored at 5 ($\circ$, $\bullet$, dashed line) or 12 °C (Δ , $\blacktriangle$) for 21 days and then transferred to 18 °C and stored for an additional 5 days. Values are means $\pm$ SE of 3 fruit. Vertical arrow denotes time at which fruit were transferred to 18 °C.

Characterization of pectins

The total uronic acid content in EIS did not change during fruit storage at 12 °C and was not affected by PG silencing, averaging $301.0 \pm 6.1 \mu\text{g mg}^{-1}$ EIS. Storage at 5 °C and subsequent transfer to 18 °C had no effect on EIS total uronic acid content, which averaged $299.2 \pm 3.7 \mu\text{g mg}^{-1}$ EIS.

Water-soluble uronic acids increased steadily during ripening of fruit stored at 12 °C from 10 to $54 \mu\text{g mg}^{-1}$ EIS in the PG-antisense fruit and from 11.5 to $100 \mu\text{g mg}^{-1}$ EIS in wild-type fruit (Figure 3-2A). CDTA-soluble pectins increased from an average of 98 to $183 \mu\text{g mg}^{-1}$ EIS during ripening of fruit stored at 12 °C and were not affected by PG levels (Figure 3-2B). Storage at 5 °C for 21 days and subsequent transfer to 18 °C for 5 days had minimal effect on the quantities of water- (Figure 3-3A) and CDTA-soluble uronic acids (Figure 3-3B), which remained at levels similar to those of mature-green fruit.

CDTA-soluble pectins showed marked molecular mass downshifts during ripening of wild-type fruit whereas depolymerization was very limited in PG antisense fruit (Figure 3-4). Low temperature storage and subsequent maintenance at 18 °C did not result in molecular mass downshift of CDTA-soluble pectins (Figure 3-5). The gel filtration profiles of water-soluble pectins were similar to those presented for CDTA-soluble pectins in both ripening and chilled fruit (data not shown).

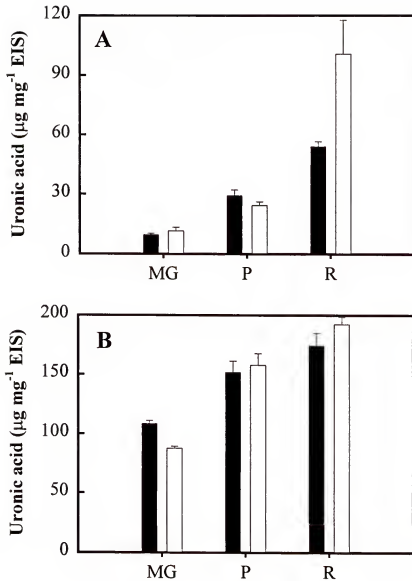


Figure 3-2. Solubility of EIS pectins in water (A) or CDTA (B) during ripening of tomato fruit at 12 °C. PG-antisense (black bars) and wild-type (white bars) fruit were analyzed at the mature-green (MG, prior to storage), pink (P, 5 days) and ripe (R, 15 days) stages. Values are means $\pm$ SE of 3 observations.

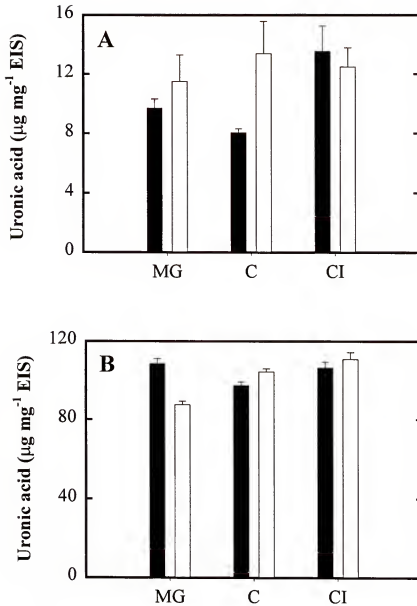


Figure 3-3. Effect of PG and chilling on the water- (A) and CDTA-solubility (B) of pectins from tomato pericarp EIS. PG-antisense (black bars) and wild-type (white bars) fruit were harvested at the mature-green (MG, prior to storage), stored at 5 °C for 21 days (chilled, C), and subsequently transferred to 18 °C for an additional 5 days to facilitate expression of injury symptoms (chill-injured, CI). Values are means $\pm$ SE of 3 observations.

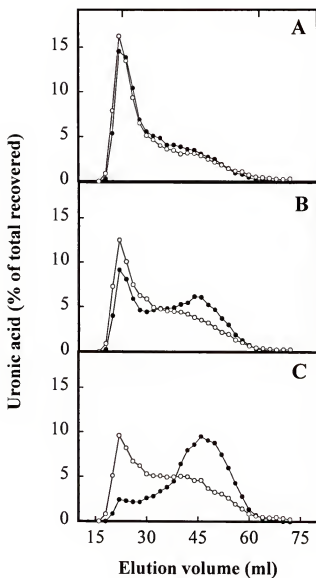


Figure 3-4. Sepharose CL-2B profiles of CDTA-soluble pectins extracted from EIS of PG-antisense (○) and wild-type (●) tomato fruit stored at 12 °C. Fruit were analyzed at the mature-green (A, prior to storage), pink (B, stored for 5 days) and ripe (C, stored for 15 days) stages.

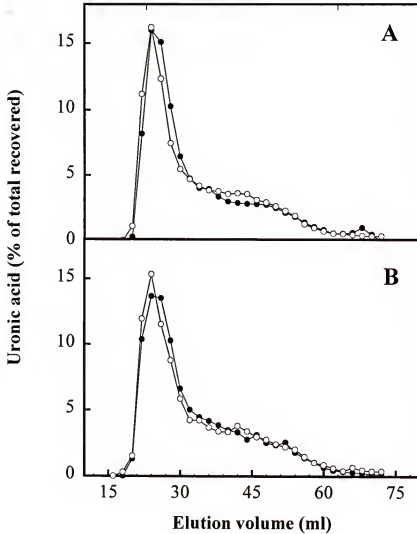


Figure 3-5. Sepharose CL-2B profiles of CDTA-soluble pectins extracted from EIS of PG-antisense (○) and wild-type (●) tomato pericarp after storage at 5 °C for 21 days (A) and after 5 days at 18 °C following storage at 5 °C (B).

Discussion

Mature-green fruit stored at 12 °C continued to develop and were fully ripe after 15 days, whereas fruit stored at 5 °C for 21 days did not ripen and developed chilling injury, as evaluated by ripening impairment, upon transfer to 18 °C. In fruit ripened at 12 °C, reduced PG levels had no effect on pericarp softening during the early stages of ripening; however, fruit with down-regulated PG were firmer than control fruit at the ripe stage, in agreement with the results of Carrington et al. (1993). Softening of pericarp upon removal of fruit from 5 °C occurred at a similar rate in wild-type and PG-antisense fruits, despite the fact that PG accumulates following, but not during, storage at 5 °C (Jackman et al. 1992, Marangoni et al. 1995).

Fruit with reduced PG levels stored at 12 °C had 46% less water-soluble pectins at the ripe stage than wild-type controls while the solubilization of pectins in CDTA was similar in PG antisense and wild-type fruit. Pectin depolymerization in antisense fruit was limited compared with wild-type. Brummell and Labavitch (1997) observed a less pronounced effect of PG. These authors found a slight delay in pectin molecular mass downshifts in the PG antisense compared with the wild-type control. Differences in the cell wall preparation method or in residual PG activity may account for the different trends.

Chilling strongly limited pectin solubilization and depolymerization, as compared with normal ripening, although softening occurred upon transfer of chilled fruit to 18 °C. PG activity does not accumulate in tomato fruits during storage at 5 °C, but accumulates upon transfer to 22 °C in a way that parallels accumulation during normal ripening

(Jackman et al. 1992; Marangoni et al. 1995). The inhibition of pectin disassembly by low-temperature storage observed in the present study contrasts with the ultrastructural evidence for middle lamella solubilization (Marangoni et al. 1989). Tomato fruit transferred from low-temperature storage to a higher temperature soften even though pectin solubility properties and molecular mass remain similar to that of mature-green fruit prior to storage, suggesting that there are different softening mechanisms in ripening and chill-injured tomato fruit. Since fruit stored at 5 °C did not accumulate PG and softening was accelerated upon transfer to 22 °C, even before PG activity could be detected, Jackman et al. (1992) speculated that softening of chilled fruit after transfer to a higher temperature was induced by nonextractable PG. In the present study, pericarp firmness, pectin solubility and molecular mass were similar in PG-antisense and wild-type fruit during storage at 5 °C or after transfer to 18 °C following low-temperature storage. Although the effects of very low levels of enzymes cannot be excluded, the data presented here indicates that PG is not the major determinant of softening of chilled fruit. Mature-green tomato fruit stored at 5 °C for 15 days had 3 times the pectinmethylesterase (PME) activity found in fruit prior to storage, which led Marangoni et al. (1995) to propose that PME is involved in chilling-induced softening through an increase in pectin hygroscopicity and swelling of the cell wall. In contrast, Jackman et al. (1992) found no correlation between firmness and the activity of PME extracted from EIS of chilled fruit. Pericarp firmness of chilled (28 days at 5 °C) tomato was also not correlated with β -galactosidase or β -glucosidase activities (Jackman et al., 1992).

Softening of chilled fruit may be determined by glycosidases or endoglycanases whose role has not been fully established. Alternatively, nonenzymic mechanisms may

play a significant role in chilling-induced softening. Increased electrolyte leakage induced by low-temperature storage (King and Ludford, 1983; Autio and Bramlage, 1986) is likely to alter the apoplastic solution. Studies of the apoplastic mineral composition in ripening (Chapter 5) and chilled (Chapter 6) tomato fruit show that the levels of cations increase (K^+) or remain constant (Na^+ , Ca^{2+} , and Mg^{2+}) during ripening but decrease when fruit stored at 5 °C for 14 days are transferred to 15 °C. Although the studies do not address the specific effects of membrane permeability and ion-exchange capacity of the cell wall on the composition of the apoplastic solution, it is likely that cell walls of chilling-injured fruit have a higher ability to immobilize cations than cell walls from ripening fruit. The immobilized cations may induce structural rearrangements in the cell wall polysaccharides that contribute to softening. However, cation immobilization does not seem to affect pectin solubilization, which remained unaltered during storage at 5 °C or subsequent transfer to 18 °C. The basis for the putative increase in cation-exchange capacity during low-temperature storage is unclear. PME has been implicated (Marangoni et al., 1995), but pectin deesterification in tomato is slower during storage at 5 °C than in fruit ripening at 22 °C (Jackman et al., 1992). The differences in the mechanisms of softening in ripening and chilled fruit may reside in different patterns of gene expression (Watkins et al., 1990) or in the effects of apoplastic solutes on enzyme activity or interactions between cell wall polysaccharides.

Summary

Softening occurs in tomato fruit during ripening and as a response to chilling. Ripening-related softening and pectin metabolism have been extensively characterized

but changes in pectins resulting from chilling injury have received limited attention. Pectin solubilization and depolymerization were examined in transgenic tomato fruit with reduced polygalacturonase (EC 3.2.1.15). Mature-green tomato (*Lycopersicon esculentum* Mill.) fruit were stored at 5 and 12 °C for 21 days and subsequently transferred to 18 °C for an additional 5 days. Pericarp firmness decreased during storage at 12 °C, and the softening rate increased after transfer to 18 °C. Ripe PG-antisense fruit were firmer than the wild-type at the same stage. Firmness remained unchanged during storage at 5 °C but decreased after transfer of chilled fruit to 18 °C. Water-soluble pectins increased 5 and 9-fold during ripening of PG-antisense and wild-type fruit, respectively, while CDTA-soluble pectins increased 0.6 and 2.2-fold in PG-antisense and wild-type fruit, respectively. Solubilization of pectins was very restricted in fruit stored at 5 °C and upon subsequent transfer to 18 °C. Water- and CDTA-soluble pectins exhibited a PG-mediated reduction in molecular mass (in Sepharose CL-2B) during ripening at 12 °C, but no depolymerization was evident in fruit stored at 5 °C, even after transfer of fruit to 18 °C for 5 days. Chilling-injured tomato fruit softened even though pectin solubilization and molecular mass distribution remained unchanged, suggesting that the softening mechanism differs in ripening and chilling-injured tomato fruit.

CHAPTER 4

COLOR, PERICARP FIRMNESS, ELECTROLYTE EFFLUX, AND PECTIN DISASSEMBLY IN RIPENING AND CHILLED TOMATO FRUIT TREATED WITH 1-METHYLCYCLOPROPENE

Introduction

In Chapter 3 pectin solubility and molecular mass were measured in ripening and chilling-injured tomato fruit in relation to pericarp tissue firmness. During ripening, pericarp tissue showed a PG-dependent increase in water-soluble pectins, a PG-independent increase in CDTA-soluble pectins, and PG-dependent downshifts in pectin molecular mass. Fruit stored at 5 °C for 21 days retained pericarp firmness. Fruit transferred to 18 °C softened at a rate similar to that of fruit ripened at 12 °C. Solubilization and molecular mass distribution of pectin from chilling injured fruit were similar to that of mature-green fruit before low-temperature storage.

Chilling injury compromises membrane competence and tissue integrity (Saltveit and Morris, 1990), inducing alterations in the apoplastic environment (Chapter 6) that may affect cell wall metabolism. PG is more influential in situations where the structural integrity of the tissue is compromised. Besides having increased firmness retention in the later ripening stages, homogenates of PG-antisense fruit have higher viscosity and higher molecular mass pectins than wild-type controls (Brummell and Labavitch, 1997;

Errington et al., 1998; Porretta et al., 1998). However, the influence of PG on pectin disassembly in chill-injured fruit has not been well documented.

Fruit stored at temperatures slightly above the injury threshold have been used as non-chilled controls in studies of the physiological effects of chilling (e. g. Jackman et al., 1992; Marangoni et al., 1995; Palma et al., 1995); however, fruit stored above the threshold temperature for injury continue to ripen, diverging developmentally from fruit stored at injurious temperatures. Alternatively, the effects of chilling have been studied by comparing the physiological characteristics of fruit after low temperature storage with those measured in fruit prior to low temperature storage (e.g., Fukushima and Yamazaki, 1978). In search of a more reliable control to assess the effects of chilling, experiments were designed using the ethylene action inhibitor 1-methylcyclopropene (MCP, Sisler and Serek, 1997) to inhibit ripening while allowing the fruit to age at a temperature above the injury threshold.

The objective of this study was to monitor firmness, electrolyte leakage, and pectin disassembly in chilled and ripening non-chilled fruit to further characterize chilling- and ripening-related softening physiology.

Material and Methods

Plant material

Tomato (*Lycopersicon esculentum* Mill.) fruit cv. Solar Set or the line 1436 (Calgene, Davis, CA) containing a PG antisense construct and its untransformed wild-type were used in these experiments. Fruit were harvested at the mature-green stage, washed in 2 mM sodium hypochlorite for 2 min, rinsed with water, and air-dried before

storage at the indicated temperatures. Fruit developmental stage was evaluated when the fruit were cut for the preparation of the cell material or for disk excision. Immature fruit were discarded.

Treatment of fruit with 1-methylcyclopropene

For MCP treatment, fruit were sealed in 19 L plastic buckets (approximately 35 fruit/bucket) containing the MCP-generator EthylBloc (BioTechnologies for Horticulture, Burr Ridge, IL). ‘Solar Set’ fruit were treated for 24 h at 15 °C with 50 mg EthylBloc dissolved in 50 mL of distilled water, yielding approximately 4.8 ppm of MCP. Fruit from line 1436 were treated for 12 h at 15 °C with 70 mg EthylBloc in 50 mL water, yielding approximately 6.7 ppm of MCP. Control fruit were maintained in sealed buckets without EthylBloc.

Storage temperatures

MCP-treated and untreated fruit were placed at 5 or 15 °C for 2 (line 1436) or 3 (‘Solar Set’) weeks, after which time the fruit at 5 °C were transferred to 15 °C. In these experiments, the removal from cold storage coincided with first evidence of red color in MCP-treated fruit stored at 15 °C. During storage, fruit were maintained in 19 L plastic buckets aerated with water-saturated air at a flow rate of approximately 20 mL min⁻¹ kg⁻¹ at 5 °C, and 75 mL min⁻¹ kg⁻¹ at 15 °C.

Firmness and color determinations

Firmness was determined on disks excised from the equatorial region of the fruit with a cork borer (15.5 mm diameter) by flat plate compression using an Instron Universal Texture Tester (model 4411) equipped with a 50 kgf load cell traveling at a crosshead speed of 50 mm min⁻¹ and a circular flat probe with 13 mm diameter. Five disks from each of 3-5 fruit were oriented with the endocarp facing up and the force required to displace the probe 3 (line 1436) or 5 mm (cv. Solar Set) was recorded. Fruit surface color was measured as described in Chapter 3.

Electrolyte leakage determination

Pericarp disks were excised from the equatorial region of each of 5 fruit (5 disks per fruit) with a 15.5 mm diameter cork borer, rinsed with distilled water, blotted dry, and weighed. The disks were incubated in 10 mL of 300 mM mannitol for 4 h in capped polypropylene tubes. Incubations were conducted at room temperature (about 24 °C) with slow shaking. Conductivity was measured with YSI conductance bridge (model 31A) equipped with a model 3403 conductivity cell (Yellow Springs, OH) immediately after addition of the bathing solution to the disks and at the end of the incubation period. The disks and bathing solution were then stored at -20 °C for at least 24 h, thawed at room temperature, and transferred to a boiling water bath for 10 minutes. The samples were cooled to room temperature in a water bath, and total electrolyte content determined by measuring the conductivity of the bathing solution.

Efflux of pectins from pericarp disks

Disks (15.5 mm diameter) from the equatorial region of 5 fruit were rinsed with distilled water, blotted dry, and incubated in 10 mL of 300 mM sucrose for 4 h at room temperature. Afterward, the bathing solution was filtered through a Whatman GF/C glass fiber filter and 40 mL of 95 % ethanol were added to the filtrate. The samples were stored at -20°C . The samples were centrifuged at 2000g for 20 min (4°C) in a Beckman centrifuge (model J2-21) equipped with a JA-20 rotor. The supernatant was discarded and the pellet washed with 40 mL of 80 % ethanol (2 X). The pellet was dissolved in 5 mL distilled water, and total uronic acids were determined by the *m*-phenylphenol method (Blumenkrantz and Asboe-Hansen, 1973) as modified by Filisetti-Cozzi and Carpita (1991).

To evaluate the effect of loss of tissue integrity on pectin solubilization and molecular mass, in one experiment disks from PG-antisense and wild-type fruit in the 300 mM sucrose solution after the 4 h incubation were stored at -20°C for at least 12 h. Afterward, the disks and solution were thawed, maintained in a boiling water bath for 20 min and pectins recovered as described above.

Pectins were concentrated by rotary evaporation at 35°C and examined on a Sepharose CL-4B column (29 cm long x 1.5 cm diameter) operated in 200 mM ammonia acetate, pH 5.0 as the mobile phase (Mort et al., 1991). The column was loaded with 500 μg of uronic acid equivalents in 1 mL aqueous solution and fractions of 2 mL were collected. Uronic acids were determined for each fraction by the *m*-phenylphenol method and expressed as a percentage of total uronic acid recovered.

Results

Color changes in ripening fruit and in response to low temperature storage

Changes in hue angle constituted the major alteration of color coordinates of fruit stored at 15 °C (Table 4-1), reflecting the transition from green to red. The chroma remained unchanged ('Solar Set') or increased (line 1436) during ripening. Both MCP and storage at 5 °C inhibited ripening, as evaluated by the development of red hue. MCP-treated mature-green fruit showed little color change for 2 (line 1436) or 3 ('Solar Set') weeks. Upon resumption of ripening, the color development of MCP-treated fruit from both cultivars was similar to that of untreated mature-green fruit.

Fruit stored at 5 °C exhibited a decline in hue angle (Table 4-2), corresponding to surface yellowing. This is evident when the color of fruit stored at 5 °C is compared with both MCP-treated fruit stored at 15 °C (Table 4-1), and fruit measured prior to cold storage (Table 4-2). L^* values remained unaltered for fruit stored at 5 °C, whereas the chroma remained constant or decreased slightly. A slight increase in lightness occurred within 24 h of transfer of fruit from 5 to 15 °C. The hue angle either remained constant or decreased slightly during exposure to 15 °C following chilling.

Pericarp firmness during ripening and in response to low-temperature storage

MCP-treated fruit (cv. Solar Set) stored at 5 °C retained pericarp firmness for the duration of the chilling exposure (Figure 4-1). MCP-treated fruit stored at 15 °C retained firmness for 3 weeks, after which time ripening resumed (Figure 4-1). Softening occurred at a comparable rate in MCP-treated fruit upon ripening at 15 °C, and chilled fruit transferred to 15 °C. Non MCP-treated fruit stored at 5 °C retained firmness during

low-temperature storage and softened after transfer to 15 °C at a rate similar to that of MCP-treated fruit under the same conditions (not shown).

Table 4-1. Effect of MCP on the surface color of tomato fruit stored at 15 °C. Values followed by the same letter (by column) are not significantly different at 5% level by the Duncan's multiple range test. Values for line 1436 are averages for PG-antisense and wild-type, since no differences were observed between the lines.

Cultivar	MCP	Days	Stage	L*	Chroma	Hue angle
Solar Set	No MCP	0	MG	57.6 a	31.8 a	118.5 a
		4	Pink	55.7 a	30.0 a	91.3 b
		9	Ripe	48.8 b	31.3 a	58.5 c
Solar Set	MCP	0	MG	56.3 a	32.3 a	118.5 a
		21	MG	58.3 a	32.1 a	116.9 a
		25	Pink	59.6 a	34.3 a	99.9 b
		29	Ripe	51.0 b	31.5 a	58.3 c
1436	No MCP	0	MG	63.0 a	29.8 c	112.8 a
		6	Pink	58.4 b	32.8 b	80.8 b
		12	Ripe	45.4 c	41.3 a	45.0 c
1436	MCP	0	MG	63.5 a	31.4 c	112.0 a
		14	MG	61.3 b	31.9 c	113.2 a
		19	Pink	62.5 ab	35.3 b	89.3 b
		22	Ripe	52.5 c	38.1 a	45.9 c

Table 4-2. Surface color of tomato fruit stored at 5 °C for 14 (line 1436) or 21 (cv. Solar Set) days and subsequently transferred to 15 °C. Values followed by the same letter (by column) are not significantly different at 5% level by the Duncan's multiple range test. Values for line 1436 are averages for PG-antisense and wild-type, since no differences were observed between the lines.

Cultivar	Days at 5 °C	L*	Chroma	Hue angle
Solar Set	0	57.0 b	32.0 a	118.5 a
	21	58.6 b	28.8 b	110.7 b
	Days at 15 °C			
	1	60.4 a	26.6 bc	110.2 b
	4	61.3 a	26.1 bc	107.4 b
	8	61.4 a	23.7 c	108.3 b
	Days at 5 °C			
	0	63.2 b	30.6 a	112.4 a
1436	14	63.9 b	32.6 a	107.8 b
	Days at 15 °C			
	1	64.8 ab	29.7 a	105.7 b
	5	65.8 a	32.4 a	101.4 c

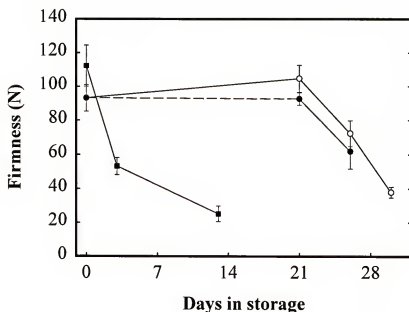


Figure 4-1. Influence of MCP and low-temperature storage on pericarp firmness of 'Solar Set' tomatoes stored at 5 or 15 °C. Mature-green fruit were treated with MCP and stored at 15 °C (○) for 30 days or 5 °C for 21 days (dashed line) and subsequently transferred to 15 °C (●) for an additional 5 days. A set of non-MCP-treated fruits was allowed to ripen at 15 °C (■).

To evaluate the effect of PG on low-temperature induced softening, the experiment was performed using PG-antisense and wild-type fruit. Softening occurred during storage at low temperature in MCP-treated fruit (Figure 4-2), as well as in non MCP-treated fruit (not shown). No significant firmness changes occurred upon transfer of chilled fruit to 15 °C. When MCP-treated fruit resumed ripening, pericarp softening occurred at a rate similar to that of untreated fruit. Fruit with reduced PG had firmer pericarp at the ripe stage and after chilling.

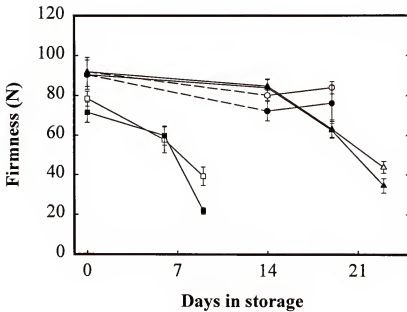


Figure 4-2. Effect of MCP, chilling and PG levels on tomato pericarp firmness. Mature-green fruit from PG-antisense (Δ , $\circ$, $\square$) and corresponding wild-type ($\blacktriangle$, $\bullet$, $\blacksquare$) were treated with MCP and stored at 15 °C for 23 days (Δ , $\blacktriangle$) or 5 °C for 14 days and subsequently transferred to 15 °C for an additional 5 days ($\circ$, $\bullet$). Non-MCP-treated fruit were allowed to ripen at 15 °C ($\square$, $\blacksquare$).

Electrolyte leakage in ripening fruit and in response to low-temperature storage

Electrolyte leakage, expressed as a percentage of total tissue electrolytes, decreased slightly or remained constant during ripening of non-MCP-treated fruit at 15 °C or upon resumption of ripening in MCP-treated fruit (Table 4-3). In some experiments, electrolyte leakage increased between the mature-green and pink stages,

followed by a decrease in ripe fruit (Table 4-3). Electrolyte efflux was not influenced by PG levels.

Expressing electrolyte leakage as percentage of total electrolytes obscures the underlying trends in effluxed and total electrolytes. The conductance of the bathing solution at the end of a 4 h incubation increased during fruit ripening, but total tissue electrolytes increased at a higher rate, resulting in a decrease in proportional electrolyte leakage (Table 4-3).

Electrolyte leakage (% of total) increased in fruit stored at 5 °C, then decreased within 24 h upon transfer of fruit to 15 °C. Leakage increased again during storage at 15 °C following chilling exposure (Table 4-4). The changes in total electrolytes during cold storage were more erratic, either increasing or remaining constant, but decreased within 24 h of transfer to 15 °C and increased during subsequent storage at 15 °C. Reduced PG levels had no influence on electrolyte leakage from pericarp disks of fruit stored at low temperature. MCP treatment induced a slightly higher electrolyte efflux (Table 4-4).

Pectin efflux from pericarp disks

In cv. Solar Set, uronic acid efflux from pericarp disks incubated in 300 mM sucrose increased more than 16-fold during ripening, from 4 $\mu\text{g}\cdot\text{g}^{-1}$ disk fresh weight to 65 $\mu\text{g}\cdot\text{g}^{-1}$ (not shown). Uronic acid efflux from MCP-treated fruit remained at levels for mature-green fruit for 3 weeks, and increased to 43.3 $\mu\text{g}\cdot\text{g}^{-1}$ as the fruit resumed ripening (Figure 4-3A).

Table 4-3. Electrolyte leakage from pericarp disks of ripening tomato fruit stored at 15 °C

Cultivar	MCP	Days	Stage	Conductance (μ mho)			Electrolyte leakage (%)
				Initial	4h	Total	
Solar Set	No MCP	0	MG	31.1 $\pm$ 5.2	182 $\pm$ 18	910 $\pm$ 64	16.4 $\pm$ 0.9
		3	Pink	22.1 $\pm$ 1.6	176 $\pm$ 12	970 $\pm$ 80	15.9 $\pm$ 0.7
		10	Ripe	33.0 $\pm$ 3.7	238 $\pm$ 17	1320 $\pm$ 98	15.7 $\pm$ 1.7
Solar Set	MCP	0	MG	31.5 $\pm$ 2.9	200 $\pm$ 15	990 $\pm$ 91	17.2 $\pm$ 0.9
		21	Green	27.6 $\pm$ 1.4	258 $\pm$ 7	1080 $\pm$ 60	21.5 $\pm$ 1.1
		27	Pink	26.3 $\pm$ 1.7	266 $\pm$ 12	1330 $\pm$ 58	18.1 $\pm$ 0.5
		30	Ripe	29.2 $\pm$ 1.7	221 $\pm$ 17	1300 $\pm$ 118	14.8 $\pm$ 0.7
PG-WT	No MCP	0	Before	15.8 $\pm$ 1.7	161 $\pm$ 10	820 $\pm$ 46	17.8 $\pm$ 1.0
		5	Pink	14.9 $\pm$ 1.7	185 $\pm$ 9	770 $\pm$ 20	22.1 $\pm$ 1.1
		12	Ripe	15.7 $\pm$ 0.8	190 $\pm$ 7	1064 $\pm$ 63	16.6 $\pm$ 1.1
PG-WT	MCP	0	MG	13.6 $\pm$ 1.5	156 $\pm$ 11	850 $\pm$ 32	16.7 $\pm$ 0.8
		14	Green	13.2 $\pm$ 0.3	167 $\pm$ 11	812 $\pm$ 45	19.2 $\pm$ 1.6
		20	Pink	11.9 $\pm$ 0.9	168 $\pm$ 10	1174 $\pm$ 61	13.3 $\pm$ 0.6
		23	Ripe	17.1 $\pm$ 1.6	199 $\pm$ 15	1174 $\pm$ 22	15.5 $\pm$ 1.0
PG-AS	No MCP	0	MG	15.0 $\pm$ 0.7	168 $\pm$ 8	870 $\pm$ 44	17.6 $\pm$ 0.2
		5	Pink	12.5 $\pm$ 0.4	187 $\pm$ 6	790 $\pm$ 29	22.1 $\pm$ 0.4
		12	Ripe	16.6 $\pm$ 1.0	186 $\pm$ 7	830 $\pm$ 44	20.6 $\pm$ 1.3
PG-AS	MCP	0	MG	24.4 $\pm$ 2.1	156 $\pm$ 6	900 $\pm$ 45	14.7 $\pm$ 0.5
		14	Green	10.6 $\pm$ 0.7	147 $\pm$ 5	900 $\pm$ 42	15.2 $\pm$ 0.5
		20	Pink	13.3 $\pm$ 0.9	175 $\pm$ 10	1190 $\pm$ 51	13.7 $\pm$ 1.0
		23	Ripe	14.0 $\pm$ 0.6	174 $\pm$ 19	1020 $\pm$ 83	15.5 $\pm$ 0.7

Table 4-4. Electrolyte leakage from pericarp disks of mature-green tomato fruit stored at 5 °C for 21 (cv Solar Set) or 14 days (line 1436) and subsequently transferred to 15 °C

Cultivar	MCP	Days	Temp (°C)	Conductance (μmho)			Electrolyte leakage (%)
				Initial	4 h	Total	
Solar Set	No MCP	0		31.1±5.2	182±18	910±64	16.4±0.9
		21	5	31.5±1.3	319±26	1130±58	25.3±1.3
		22	15	29.6±1.4	233±24	1063±75	18.9±1.3
		26	15	24.4±2.7	264±19	1175±63	20.7±2.2
Solar Set	MCP	0		31.5±2.9	200±15	990±91	17.2±0.9
		21	5	32.1±5.1	329±24	1060±66	28.1±1.5
		22	15	28.4±3.0	296±30	1055±55	25.2±1.5
		26	15	26.6±3.6	326±11	1290±73	23.6±1.8
		30	15	22.0±2.1	440±54	1430±58	29.5±4.4
PG-WT	No MCP	0		15.8±1.7	161±10	820±46	17.8±1.0
		14	5	12.0±1.3	170±18	940±68	16.9±1.4
		15	15	11.1±1.8	121±10	746±54	14.8±0.9
		19	15	9.3±0.4	135±7	988±63	12.8±0.6
PG-WT	MCP	0		13.6±1.5	156±11	850±32	16.7±0.8
		14	5	11.8±0.6	165±12	830±30	18.4±1.0
		15	15	14.3±1.1	139±3	796±40	15.8±0.7
		19	15	14.3±0.9	154±6	995±71	14.2±0.8
PG-AS	No MCP	0		15.0±0.7	168±8	870±44	17.6±0.2
		14	5	15.7±0.8	139±7	810±33	15.2±0.6
		15	15	9.6±0.5	121±12	752±88	14.9±0.4
		19	15	13.5±1.5	154±5	1040±53	13.6±0.8
PG-AS	MCP	0		24.4±2.1	156±6	900±45	14.7±0.5
		14	5	15.6±1.5	172±22	942±75	16.4±1.2
		15	15	10.7±0.9	136±5	776±29	16.1±0.1
		19	15	10.8±0.9	136±8	980±55	12.8±0.7

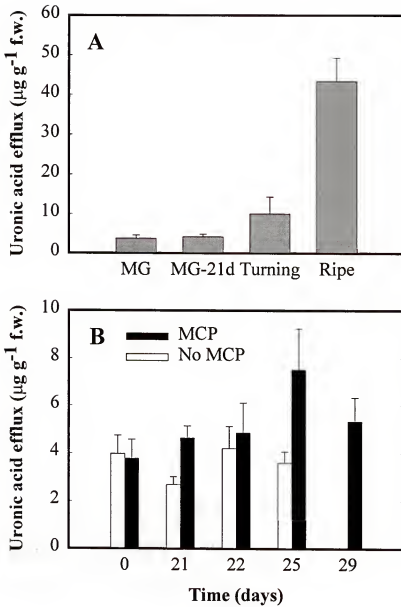


Figure 4-3. Pectin efflux from pericarp disks of 'Solar Set' tomato. MCP-treated fruit stored at 15 °C (A) remained green (MG 21d) for 21 days and then resumed ripening. Fruit stored at 5 °C for 21 days and then transferred to 15 °C for an additional 8 days (B).

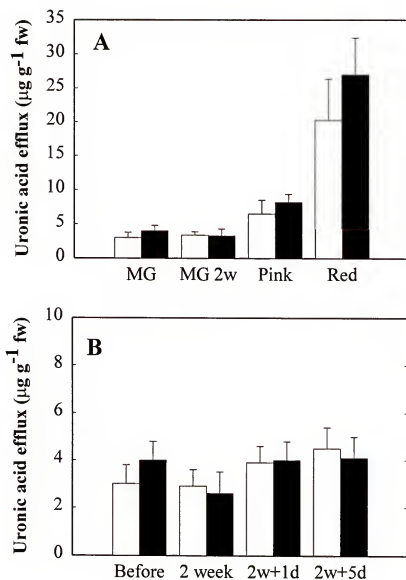


Figure 4-4. Pectin efflux from pericarp disks of MCP-treated fruit from PG-antisense (white bars) and wild-type (black bars) lines. Fruit stored at 15 °C (A) were inhibited from ripening for 14 days and then resumed ripening. Fruit stored at 5 °C (B) for 14 days were then transferred to 15 °C and stored at this temperature for an additional 5 days.

The release of uronic acids from the PG-antisense and wild-type lines followed a trend during ripening similar to that observed for cv. Solar Set. Efflux from mature-green fruit was $2\text{--}4\ \mu\text{g}\cdot\text{g}^{-1}$ in both lines. Efflux from wild-type fruit was slightly higher than from the antisense, although differences were not significant. Both PG-antisense and wild-type fruit treated with MCP and held for 2 weeks at $15\ ^\circ\text{C}$ showed no increase in uronic acid efflux (Figure 4-4A).

Uronic acid efflux did not change in fruit held for 2-3 weeks at $5\ ^\circ\text{C}$, and little or no change was observed after transfer to $15\ ^\circ\text{C}$ for 5 days (Figures 4-3B and 4-4B). Efflux of uronic acid from fruit exposed to low temperature was $3\text{--}5\ \mu\text{g}\ \text{g}^{-1}$, similar to the levels found in mature-green fruit. MCP did not affect the efflux of uronic acid from fruit exposed to low temperature (Figures 4-3B and 4-4B). Antisense reduction of PG had no effect on uronic acid efflux during storage at $5\ ^\circ\text{C}$ or upon transfer to $15\ ^\circ\text{C}$ (Figure 4-4B).

Molecular mass distribution of the pectins released from pericarp disks

Pectins released from pericarp disks of mature-green fruit had a high degree of polymerization, eluting in the void volume of the Sepharose CL-4B column. Pectins released from pericarp disks excised from fruit stored at $5\ ^\circ\text{C}$ for 2 weeks and then maintained at $15\ ^\circ\text{C}$ for 5 days were similar in mass distribution to those from mature-green fruit (Figure 4-5A). Fruit treated with MCP remained green for 2 weeks and

showed the same pattern of pectin molecular weight distribution as the mature-green fruit (Figure 4-5B). During ripening, pectins became more polydisperse, with a marked downshift in molecular weight distribution of pectins from wild-type fruit, whereas limited depolymerization was observed in the PG-antisense (Figure 4-5D).

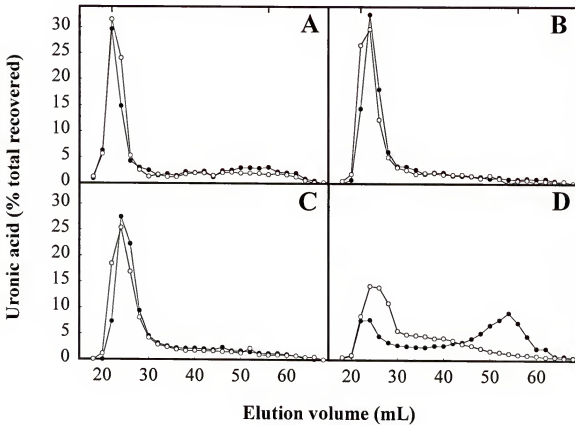


Figure 4-5. Sepharose CL-4B profiles from pectins released from PG-antisense (○) or wild-type (●) pericarp disks incubated in an 300 mM sucrose. A- Mature-green fruit; B- MCP-treated fruit after storage at 15 °C for 14 days; C- Chilling-injured fruit after 5 days at 15 °C following storage at 5 °C for 14 days; D- Ripe fruit. Tick marks at the top of the figure indicate void volume (left) and included volume (right).

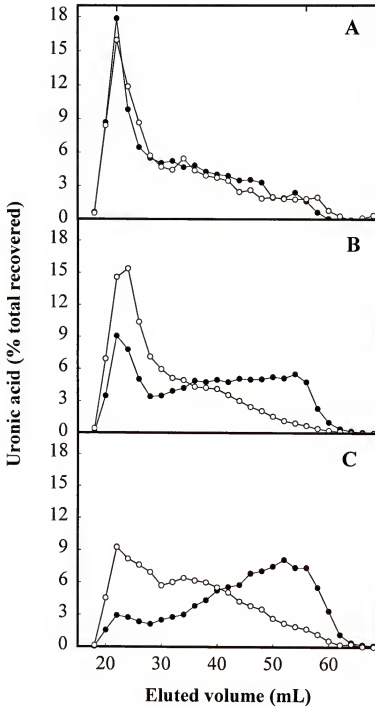


Figure 4-6. Sepharose CL-4B profiles of pectins released from PG-antisense (O) and wild-type (●) pericarp disks incubated in 300 mM sucrose after disruption of the tissue and described in materials and methods. Fruit stored at 5 °C for 21 days (A), stored at 5 °C for 21 days and subsequently transferred to 15 °C for 5 days (B), and ripe fruit stored at 15 °C (C). Tick marks at the top of the figure indicate void volume (left) and included volume (right).

When the pericarp tissue of fruit allowed to express injury symptoms at 15 °C after storage at 5 °C was disrupted by freezing and thawing after incubation in 300 mM sucrose, a clear effect of PG levels on the molecular mass distribution of pectins became apparent (Figure 4-6B). No PG effect was observed immediately after the 2-week low temperature storage (Figure 4-6A). In ripe fruit stored at 15 °C, pectins released from disrupted tissue were extensively depolymerized in wild-type fruit, whereas limited breakdown had occurred in antisense fruit (Figure 4-6C).

Discussion

The differential effect of storage temperature on the metabolism of chilling sensitive fruit has been an inherent problem in designing experiments to study the effects of chilling, leading to confounding of the effects of temperature, ripening, and chronological aging. MCP treatment was effective in maintaining pericarp color, firmness, and pectin solubilization and depolymerization at the levels found in mature-green fruit. These physiological characteristics were not affected by aging; "time zero" controls are thus validated by the data from MCP-treated fruit.

Chilling injury causes uncharacteristic coloration in many fruits. A failure to develop the coloration of ripe fruit is one of the symptoms of severe chilling injury in tomatoes (Dodds et al., 1991; Jackman et al., 1992; Côté et al., 1993). In the present study, the most consistent effect of chilling on the surface color of tomatoes was a slight decrease in the hue angle during storage at 5 °C, corresponding to yellowing. No further

yellowing occurred when fruits from the cv. Solar Set were transferred to 15 °C after 3 weeks at 5 °C. However, some development of yellow hue continued in line 1436 following transfer to 15 °C after 2 weeks at 5 °C, without ever developing red color. Yellowing during the low temperature exposure is unrelated to ripening and occurs at 5 °C but not at 2 °C (Dodds et al., 1991) and reflects the degradation of chlorophylls, associated with impaired synthesis of lycopene (Bergevin et al., 1993). Hue angle, and to a lesser extent L^* were the best coordinates for characterizing the effects of chilling on tomato color. The a^* value has also been used for the same purpose (Côté et al., 1993; Jackman et al., 1992; Nussinovitch et al., 1996), but it does not describe the yellowing that occurs during chilling.

Softening has been recognized as a symptom of chilling injury in tomatoes (Hobson, 1987). Pericarp of tomato fruit retained its firmness during storage at low temperature but softened upon transfer to a permissive temperature at a rate similar to that of fruit during normal ripening. In one experiment, softening occurred during the low temperature treatment and firmness was retained upon removal to 15 °C (Figure 4-2). Maintenance of pericarp firmness during storage at 5 °C for 28 days was observed by Jackman et al. (1992), whereas Jackman et al. (1990) and Marangoni et al. (1995) reported the occurrence of softening under similar conditions. The different behaviors could be due to different rates of water loss.

As reported in Chapter 3 fruit with down-regulated PG were firmer than the wild-type fruit at the full-ripe stage. Softening upon removal from low temperature storage also occurred at a faster rate in wild-type than in PG-antisense fruits, in consonance with

the fact that PG accumulates following, but not during cold storage (Jackman et al., 1992; Marangoni et al., 1995).

Analysis of cell wall pectins is typically performed in ethanol or acetone extracts of fruit tissue. These preparations are obtained by means that are likely to wash away some portion of the apoplastic calcium (Huber, 1991). To circumvent the effects of the cell wall isolation protocol on pectin solubilization, pectin efflux from disks of pericarp tissue incubated under isotonic conditions (300 mM mannitol) was examined. The release of uronic acid from disks from chilled fruit differs from the pattern observed during ripening. The efflux from ripe pericarp disks was much higher than from chilled fruit indicating that pectin catabolism was slowed by the low-temperature exposure. This effect persisted 5 days after removal to 15 °C. Fruit with down regulated PG activity released less pectin than wild-type fruit, but the difference between both lines was more accentuated in fruit allowed to ripen normally than in fruit submitted to the low temperature treatment. Moreover, PG levels had a significant effect on the water-solubility of ripe fruit pectins (Chapter 3), yet the effect on pectin efflux from pericarp disks was much smaller, indicating that pectins solubilization *in situ* may be limited.

The molecular mass distribution of water- and CDTA-soluble pectins from mature-green PG-antisense and wild-type fruit were similar and showed no molecular mass downshift after exposure to low temperature or upon transfer to 15 °C for up to 5 days (Chapter 3). Similarly, pectin passively released from disks into an isotonic bathing solution showed no depolymerization in chilling injured fruit (Figure 4-5B and C). However, when the tissue was disrupted after incubation, there was a molecular mass downshift in pectins from wild-type fruit held at 15 °C for 5 days after a 3- week

exposure to low temperature. This effect was not apparent after 3 weeks at 5 °C. The disparity between the profiles of passively effluxed pectins and the pectins released upon tissue disruption (by freezing, thawing and boiling) in the wild-type and antisense fruit suggests that PG activity is strongly limited in chilling injured fruit. Fukushima and Yamazaki (1978) attributed the decreased solubility of pectins in chilled fruit to demethylation, and extractable PME activity was shown to increase during cold storage (Fukushima, 1978; Marangoni et al., 1995). However, pectin demethylation also occurs during fruit ripening (Koch and Nevins, 1989) and that does not prevent increased pectin solubilization (Figure 4-3A).

Huber and O'Donoghue (1993) have proposed that *in vivo* restrictions to PG activity may be imposed by apoplastic pH and ionic composition. In support of this proposal, alteration of the apoplastic environment in overripe or damaged fruit favors PG catalysis (Schuch et al., 1991; Brummell and Labavitch, 1997). More recently Chun and Huber (1998) showed that PG activity is strongly modulated by the pH and mineral composition anticipated for tomato fruit apoplast. The increase in electrolyte leakage that is often induced by chilling (King and Ludford, 1983; Autio and Bramlage, 1986; King and Ludford, 1983) should induce drastic alterations of the apoplastic environment. Our results show that electrolyte leakage increased in fruit stored at low-temperature, but not in fruit ripening at 15 °C, suggesting that alterations in the apoplastic pH and mineral composition may be distinct in ripening and chilling-injured fruit. The fact that pectin solubilization in pericarp disks was very restricted in chilled fruit as compared with ripening fruit and that a PG effect was only evident when the tissue was disrupted, suggests that chilling-induced damage does not involve loss of compartmentation.

Moreover, eventual changes in the solute composition of the apoplast are likely to be restrictive of PG activity. Cell wall metabolism in chilled fruit is different from that of ripening fruit, possible due to different patterns of alteration in the apoplastic pH and ionic strength.

Summary

Textural changes in fruit are associated with both ripening and chilling injury. Cell wall disassembly, especially evident in the middle lamella, is considered to be the main mechanism underlying the textural changes. This study addressed the role of low-temperature storage and polygalacturonase on tomato pericarp firmness, pectin solubilization, and depolymerization. Fruit treated with 1-methylcyclopropene (MCP) were used as non-ripening controls to evaluate the effects of chilling. Mature-green fruit treated with MCP and stored at 15 °C retained color, firmness, and showed no change in pectin solubilization for 2 to 3 weeks, after which time ripening resumed. Low-temperature storage reduced surface hue, but did not affect chroma or lightness. Pericarp firmness remained constant or declined slightly during storage at 5 °C, but rapid softening often occurred upon subsequent transfer of previously chilled fruit to 15 °C. Pectin solubilization and depolymerization increased during fruit ripening, but were strongly limited in fruit that had been stored at 5 °C, even after 5 days of subsequent storage at 15 °C. PG levels had minimal effect on passive efflux of pectins from pericarp disks. Fruit with reduced PG levels retained a higher proportion of high molecular mass pectins at the ripe stage than the wild-type. Cold storage inhibited the PG-mediated breakdown of pectins, and the effect persisted even upon subsequent transfer of chilled fruit to higher

temperatures. Inhibition of pectin breakdown was overcome by disrupting the tissue. These results suggest that chilling alters the mechanism of fruit softening and cell wall metabolism. PG activity *in vivo* is more limited in chilling injured than in ripe tomato fruit possibly due to differences in the apoplastic environment.

CHAPTER 5

APOPLASTIC pH AND INORGANIC ION LEVELS IN TOMATO FRUIT: A POTENTIAL MEANS FOR REGULATION OF CELL WALL METABOLISM DURING RIPENING

Introduction

Apoplastic conditions contribute to many aspects of plant development. In auxin-mediated extension growth of vegetative organs, for example, apoplastic pH exerts a strong influence on turgor and wall loosening, possibly via control of hydrolytic reactions and intermolecular interactions between structural carbohydrates and proteins (Rayle and Cleland, 1992). The import of photoassimilates into rapidly growing sinks, including tomato fruit, can follow an apoplastic route involving cell wall invertase and plasma membrane transporters. Glucose uptake by tomato fruit protoplasts is partially inhibited by H^+ -ATPase inhibitors and K^+ -ionophores (Brown et al., 1997). The apoplastic environment also influences reactions involved in elicitor metabolism and pathogenesis (De Lorenzo et al., 1991; Knogge, 1996).

The dynamic changes in the cell wall of ripening fruit are anticipated to be under tight control by local pH and ionic conditions; however, relatively few studies have addressed these interactions. Several researchers have proposed a role for apoplastic pH in regulating fruit textural changes (Knee, 1982; Ugalde et al., 1988; Huber and O'Donoghue, 1993; Heyes et al., 1997). Knee (1982) proposed apoplastic acidification as

a means for explaining the solubilization of pectins (via calcium displacement) in ripening pome fruits. Other researchers have pointed out that apoplastic conditions might influence the catalytic behavior or activity of cell wall hydrolases (Huber and O'Donoghue, 1993), which as a group show a wide range of pH optima and sensitivity to ionic conditions. Theoretical models and in vitro studies have shown that binding and catalytic behavior of several cell wall enzymes are altered in response to changes in pH, ionic composition and ionic strength (Ricard et al., 1981; Crasnier et al., 1985; Ricard and Noat, 1986; Nari et al., 1986). Ionic composition and strength can influence protein binding and kinetics due to shielding effects on the charges of the protein and on polyelectrolytic substrates.

Numerous studies have addressed the influence of pH and ionic conditions on the activities of wall hydrolases purified from ripening fruits; however, these characterization studies were concerned largely with establishing conditions for achieving maximum catalysis in vitro. Whether these reaction conditions represent the environment of the apoplast in ripening fruits is unknown, but unlikely considering the rather high ionic strengths employed in these studies.

Ruan et al. (1995) reported a pH of near 6 for the pressure-exuded sap of tomato fruit harvested during the active growth phase (25 days postanthesis). Using equilibrium techniques, Ugalde et al. (1988) reported that intercellular pH declined from near 7.0 to 4.5 for ripening peach and apricot. The mineral composition of fruit apoplast has been estimated at specific developmental stages (MacDougall et al., 1995; Ruan et al., 1996) but there is little information on the dynamics of the apoplastic environment during ripening. Expectations that the apoplastic environment changes with ripening are

warranted. Numerous studies have reported increases in net ion efflux from tissues of ripening fruits and other senescing organs (reviewed in Marangoni et al., 1996). Changes in ion efflux patterns may reflect programmed alteration of the cell wall environment.

Although probing the apoplastic compartment without disturbing its native state has proven difficult (Grignon and Sentenac, 1991), Ruan et al. (1995) provided strong evidence for the apoplastic origin of a pressure-extracted fluid from immature tomato fruit. The objective of the present study was to determine the dynamics of apoplast pH and mineral composition during the ripening of tomato fruit.

Material and Methods

Plant material

Tomato (*Lycopersicon esculentum* Mill.) fruit of fresh market line 1436 (obtained from Calgene, Davis, CA) containing an antisense construct for polygalacturonase and its parental wild-type were grown at the Univ. of Florida Horticultural Unit, Gainesville, during the spring of 1997. Fruit were harvested at the immature or mature-green stages and stored at 12 °C and 90 % relative humidity to ripen.

Extraction of apoplastic and bulk sap

Apoplastic fluid was extracted using a pressure bomb (Ruan et al., 1995). Fruits were inserted in a rubber balloon and placed inside a pressure chamber with the equatorial region facing up. A 23-gauge hypodermic needle connected to a 0.8-mm inner diameter Teflon tube was inserted approximately 2 mm into the pericarp at the equatorial region of the fruit, and the tube passed to the exterior of the chamber through a rubber

stopper. The pressure was increased using nitrogen at a rate of about 0.01 MPa s^{-1} until fluid was visible in the tube. The fluid was extracted from immature and mature-green fruit at a constant pressure of about 0.6-0.7 MPa whereas pressures of 0.3-0.5 MPa were used for pink and red fruit. The first 20 μl of fluid were discarded and the next 50 to 100 μl were collected in a polypropylene vial and used for analysis.

After extraction of fluid, the fruits were cut to ascertain the location of the needle in the pericarp wall and the fruit maturity (immature vs. mature-green). The locule and pericarp tissues were excised and stored at -30°C . Bulk sap was obtained by thawing and centrifuging the tissue, and then collecting the supernatant.

Osmolality, pH, ionic concentration and conductivity of apoplastic fluid

Osmolality was measured with a Wescor vapor pressure osmometer (model 5500, Logan, UT) using 10 μl of the pressure-extracted fluid. Osmotic potential was calculated considering $1.0 \text{ Osmol kg}^{-1} = 2.48 \text{ MPa}$ (Schmalstig and Cosgrove, 1988).

A Lazar microelectrode (model PHR-146, Los Angeles, CA) was used to measure pH. K^{+} , Na^{+} , Ca^{2+} , Mg^{2+} , and P concentrations were determined by inductively coupled plasma spectroscopy (model ICAP 9000, Thermo Jarrell Ash Corporation, Franklin, MA). Chloride was measured by an automated colorimetric procedure using an Alpkem RFA-300 spectrophotometer (Wilsonville, OR). Electrical conductivity was measured with a Markson EC meter (model 1054, Hillsboro, OR). Since no differences in the measured variables were observed between the polygalacturonase antisense and wild-type, values are presented averaged over both lines.

Results

Extraction of apoplastic fluid

To collect putative apoplastic fluid, the pressure applied to each fruit was increased until a clear sap was visible in the tube, at which time the pressure was held constant throughout the collection period. Fruits were typically pressurized for 15-30 min to collect 50 to 100 μ l of exudate. In immature and mature-green fruits, initial sap flow occurred at about 0.6-0.7 MPa, values in close agreement with the pressure reported by Ruan et al. (1995) for extraction of apoplastic sap from immature tomato fruit. Lower pressures (0.3-0.5 MPa) were effective at expelling fluid from pink and ripe fruit. On occasion, we observed that flow initiated immediately on insertion of the needle, continuing for some time in the absence of applied pressure. In these instances, we discovered that the needle had incidentally traversed into the locule cavity, facilitating a mass flow of locular liquid. Interestingly, exudation required penetration only of the locular chamber, and did not reflect penetration of or injury to locular tissues. Further investigation revealed that the magnitude of this positive locule pressure changed during fruit development. Negligible in mature-green fruit, the pressure increased during ripening, reaching a maximum at the pink stage and thereafter subsiding to preclimacteric values (data not shown). For this reason, bulk locule sap was included as an additional control to address possible contamination of the pericarp apoplastic samples by infiltration of the locule liquid driven by the internal pressure.

As an additional measure of the effects of pressure on fruit integrity, mature-green and ripe fruit were pressure treated (0.9 MPa, 45 min) and then stored at 15 °C. As evaluated by color and overall appearance of the pericarp and locule tissues, the

subsequent ripening of mature-green fruit was not impaired by pressure treatment. Similarly, ripe fruit remained intact after pressure treatment and continued to soften and senesce for up to 5 days, with no visible evidence of damage.

Osmolality of the apoplastic and bulk tissue fluids

To ascertain the possibility that pressure-extracted sap was contaminated with fluid of protoplasmic origin, osmolality of the putative apoplastic fluid was compared with that of liquid recovered from freeze-thawed pericarp and locule tissues.

Osmolality of apoplastic and bulk fluids from tomato fruit at 4 stages of development are shown in Figure 5-1. The osmolality of the pressure-extracted fluid was 25-35% of values for bulk pericarp sap at the immature and mature-green stages, increasing to 45-50% during ripening. The differences between the osmolality of the pressure-extracted fluid and the bulk sap from both the locule and the pericarp, along with differences in the mineral levels (presented below), attest to its apoplastic origin.

Pressure-extracted samples with osmolality values averaging about one-third of those measured for the bulk pericarp fluid were considered as reasonably free of protoplasmic contamination and used for pH and mineral determinations.

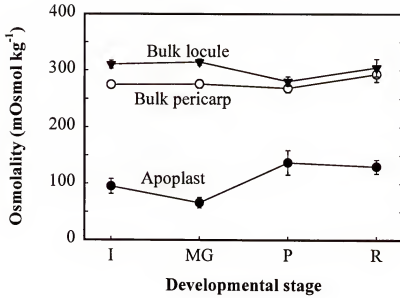


Figure 5-1. Osmolality of the pressure extracted fluid and the bulk pericarp and locule tissues at different maturity stages: I, immature; MG, mature-green; P, pink; R, ripe. Error bars represent $\pm$ SE of 6 observations.

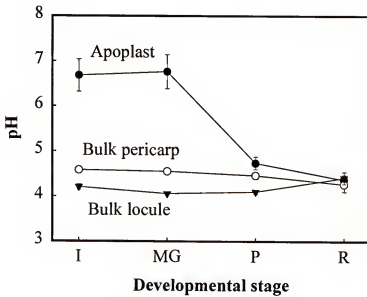


Figure 5-2. pH of the apoplastic and bulk pericarp and locule tissues at different maturity stages. Maturity stages as described in Figure 5-1. Error bars represent $\pm$ SE of 6 observations.

pH of apoplastic and bulk fluids

The pH of the pressure-extracted fluid from immature and mature-green tomato fruits averaged 6.7, declining to about 4.8 in pink fruit and 4.4 in red fruit (Figure 5-2). The bulk sap of pericarp and locule tissues averaged about 4.5 and 4.1, respectively, and showed little variation during the developmental period measured.

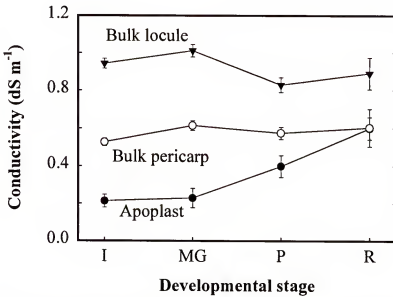


Figure 5-3. Electrical conductivity of apoplastic and bulk pericarp and locule saps at different maturity stages. Maturity stages as described in Figure 5-1. Error bars represent $\pm$ SE of 6 observations.

Electrical conductivity of apoplastic and bulk fluids

Conductivity values were measured in apoplastic and bulk solutions diluted 100- and 10-fold, respectively. Although conductivity does not correlate in a linear fashion with concentration, the assumption of linearity is a close approximation for dilute solutions. Accordingly, dilution factors were adjusted to yield apoplastic- and bulk-derived solutions of low and comparable conductivities.

The conductivity of apoplastic fluid increased 3-fold during ripening (Figure 5-3). In immature and mature-green fruit, apoplastic conductivity was about 40% of the value for the bulk pericarp, increasing to values similar to those of the bulk sap at the fully ripe stage.

Inorganic mineral composition

The contribution of minerals to the osmolality was more variable in the apoplast than in the bulk tissues. The inorganic ions analyzed accounted for from 37-58% of the total solutes in the apoplastic fluid, and only 23% and 30% of the solutes in the bulk liquids of pericarp and locule tissues, respectively.

Potassium was the most abundant ion in both apoplastic and bulk saps (Figure 5-4A). $[K^+]$ in bulk fluids remained relatively constant during ripening, averaging 47 mM and 76 mM, respectively, for pericarp and locule tissues. Apoplastic $[K^+]$ averaged 13 mM at the immature and mature-green stages and increased to 37 mM during ripening (Figure 5-4A). The levels of Na^+ in the apoplastic sap were about 6.5 mM, 5.5 to 7.5-fold higher than levels in the bulk pericarp, and showed no change during ripening (Figure 5-4B). The concentration of Na^+ in the bulk locule sap decreased from 2.4 to 1.1 mM during ripening.

Apoplastic Ca^{2+} and Mg^{2+} did not show a clear pattern of variation during ripening (Figure 5-5). Concentrations in the apoplast were 16-27 and 3 times higher than in the pericarp bulk fluid for Ca^{2+} and Mg^{2+} , respectively. The differences between apoplastic and bulk Ca^{2+} and Mg^{2+} levels were maintained throughout ripening.

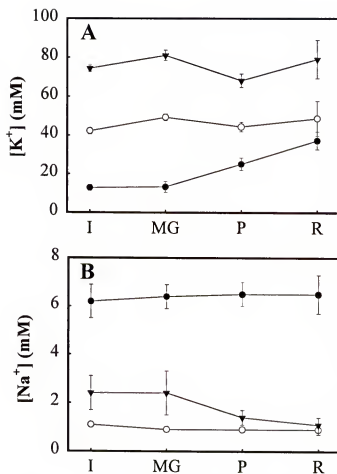


Figure 5-4. Concentration of monovalent cations in the apoplastic solution and in the bulk fluids from pericarp and locule tissues. Potassium (A) and sodium (B) levels are presented for the apoplastic fluid (●) and bulk saps of the pericarp (○) and locule (▼) tissues. Maturity stages as described in Figure 5-1. Error bars represent $\pm$ SE of 6 observations.

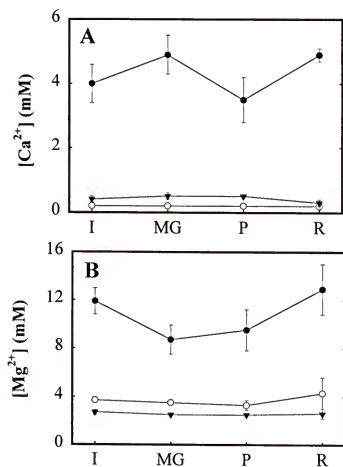


Figure 5-5. Concentration of divalent cations in the apoplastic solution and in the bulk fluids from pericarp and locule tissues. Calcium (A) and magnesium (B) levels are presented for the apoplastic fluid ($\bullet$) and bulk saps of the pericarp ($\circ$) and locule ($\blacktriangledown$) tissues. Maturity stages as described in Figure 5-1. Error bars represent $\pm$ SE of 6 observations.

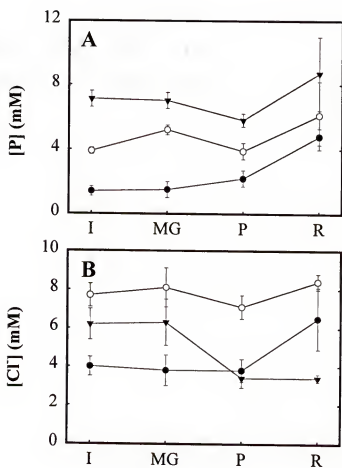


Figure 5-6. Concentration of inorganic anions in the apoplastic solution and in the bulk fluids from pericarp and locule tissues. Phosphorus (A) and chloride (B) levels are presented for the apoplastic fluid (●) and bulk saps of the pericarp (○) and locule (▼) tissues. Maturity stages as described in Figure 5-1. Error bars represent $\pm$ SE of 6 observations.

The level of anions in the apoplast (assuming most of the P is present as inorganic phosphate) remained relatively constant until the mature-green and pink stages for P and Cl^- , respectively. The level of P increased more than 3-fold, from 1.5 to 4.8 mM during ripening, whereas Cl^- increased 70 % from 3.8 to 6.5 mM between the pink and ripe stages (Figure 5-6). The levels of P in the apoplast were 30-40% of the levels in the bulk pericarp sap in immature and mature-green fruit, whereas the concentration of apoplastic Cl^- was about 50% of the bulk values. The apoplastic concentrations of P and Cl^- collectively increased to about 80% of the levels in the bulk pericarp at the ripe stage.

Discussion

These studies provide evidence that the apoplast of ripening tomato fruit undergoes dynamic changes during ripening, exhibiting a marked decline in pH and increase in the levels of some ions, particularly K^+ .

The values for apoplastic osmolality reported here are similar to those for young, immature tomato fruit (Ruan et al., 1996). These authors obtained apoplastic fluids of about 100 mOsmol kg^{-1} and pericarp bulk fluids of 280 mOsmol kg^{-1} . In the present study, the osmotic potential for the bulk pericarp tissue decreased from -0.68 MPa in immature and mature-green fruits to -0.73 MPa in red fruit. The changes in osmotic potential were greater for the apoplastic fluids, increasing from -0.24 MPa in immature fruit to -0.16 MPa in MG, and then decreasing to -0.34 and -0.32 MPa in pink and red fruit, respectively. A redistribution of osmotically active solutes to the apoplast would

decrease the osmotic potential in this compartment and cause a decrease in turgor pressure during ripening. Consistent with this idea, a decrease in turgor has been reported for ripening tomato (Shackel et al., 1991) and strawberry (Pomper and Breen, 1995) fruits. Although the total minerals and protons in the apoplast increased several fold during ripening, resulting in a 3-fold increase in conductivity, the concentration of osmotically active solutes in the collected sap increased only by a factor of 2. The discrepancy between ion concentration and osmolality may be caused by condensation of cations to neutralize the negative charges being generated in the cell wall. Condensed ions have negligible effects on the osmolality of the solution (McLaughlin, 1989).

The apoplastic sap of immature and mature-green tomato has an average pH of 6.7, decreasing to 4.4 in red fruit. Using an equilibration method to estimate apoplastic pH, Ugalde et al. (1988) observed similar trends for ripening peach and apricot fruits. Prior to ripening (Stage II), peach and apricot mesocarp exhibited apoplast pH values near 7. During ripening (Stage III), apoplastic pH dropped to 4.3 in peach and 3.4 in apricot. Ballarin-Denti and Antoniotti (1991) found an apoplastic pH of 7.8 for squash fruit, although the authors gave no indication of the developmental stage of the fruit. Collectively, these studies indicate that the apoplastic pH of pre-ripe fruit approaches neutrality, and decreases as much as 3 units during ripening. The pH of the bulk pericarp and locule tissues is in the range reported for several tomato cultivars (Stevens et al., 1977, Al-Shaibani and Greig, 1979).

The pH of fruit apoplast seems to be actively regulated. Incubation of fruit tissues in solutions of pH values different from the apoplastic pH results in reequilibration of the system at or near the intercellular pH (Ugalde et al., 1988; Ballarin-Denti and Antoniotti,

1991). The mechanism for the change and homeostasis of the apoplastic pH may reside in plasmalemma ATPase activity. ATPase activity has been reported to increase during ripening of several fruits, including the climacteric apple (Lurie and Ben-Arie, 1983) and pepino (Heyes et al., 1997), and the non-climacteric strawberry (Ben-Arie and Faust, 1980).

Some evidence has been presented for the breakdown of compartmentation between the symplast and apoplast during ripening of grape berry (Lang and Düring, 1991) and strawberry (Pomper and Breen, 1995). Ripening-related changes in membrane permeability, caused by lipid catabolism and oxidative stress (Ferrie et al., 1994; Rogiers et al., 1998) might permit the equilibration of protons and K^+ between the cytosol and the apoplastic free space. The fact that the pH of the apoplastic and bulk solutions converged in ripe fruit while the osmolality of these fluids remained significantly different favors a controlled efflux of protons and K^+ rather than a disruption in compartmentation.

The conductivity of the apoplastic fluid increased 3 fold during ripening paralleling the increase in apoplastic K^+ and H^+ . The total amount of solutes (osmolality) in the apoplastic solution doubled during ripening. The bulk conductivity of locular tissue was about 50 to 80 % higher than that of the pericarp tissue, although the osmolality of the locule was just 3 to 15 % higher.

Averaged over all ripening stages, the inorganic minerals K^+ , Na^+ , Ca^{2+} , Mg^{2+} , Cl^- and P accounted for 48 % of the osmolality of the apoplastic fluid and 30 % of the bulk pericarp. Ruan et al. (1996) found that inorganic ions were responsible for 43 % of the total osmolality of the apoplastic fluid of immature tomato fruit and 27% of the bulk tissue osmolality.

The 3-fold increase in apoplastic $[K^+]$ during ripening could be accounted for by passive diffusion from the symplastic pool due to increased membrane permeability, or by altered activity of ion channels. Several ion channels respond to changes in the potential difference across the membrane and are likely to be tuned for the specific physiology of the tissue in which they are present (Tester, 1990). A mechanism involving voltage-dependent outward rectifying K^+ channels could be responsible for the increase in apoplastic K^+ levels during ripening. These channels are activated by depolarization of the plasma membrane (Hedrich and Schroeder, 1989) and have been implicated in membrane repolarization of several cell types (Moran et al., 1986; Hedrich and Schroeder, 1989). As ripening proceeds, the cell wall pectic polymers are de-esterified (Koch and Nevins, 1989), increasing the negative charge density in the apoplast. This condition may alter the charge gradient across the membrane, activating K^+ efflux by voltage-regulated channels. Interestingly, K^+ efflux has been implicated in programmed cell death in both plant (Atkinson et al., 1990) and animal systems (Bortner et al., 1997). The observed increase in K^+ efflux during fruit ripening may be an expression of the senescence program.

The mineral levels in the apoplastic solution of immature fruit in the present study were different from the values reported by Ruan et al. (1996). The most striking differences were 10-12 times higher levels of Na^+ , twice the Mg^{2+} , and about 50% of the P and Cl^- in our samples. Differences in soil, climate, cultivar, developmental stage and cultural practices may contribute to these discrepancies. The concentrations of these minerals in the bulk pericarp were similar (except for Mg^{2+} , almost twice as high in our

samples), suggesting that the apoplastic mineral composition is more affected by environmental variation than is the symplastic compartment.

The levels of Ca^{2+} and Mg^{2+} in pressure-exuded apoplastic sap did not show a clear trend during ripening. The apoplastic levels of Ca^{2+} reported here (4.0-4.9 mM) and the even higher values (5.6-6.9 mM) previously reported for immature tomato fruit (Ruan et al., 1996) are sufficient to strongly limit pectin solubilization from isolated cell walls (Rushing and Huber, 1987). Due to electrostatic effects, the ionic environment of the polyanionic matrix is likely to be substantially different from the apoplastic free solution (McLaughlin, 1989; Grignon and Sentenac, 1991). The pressure-extracted fluid is probably representative of the free solution, underestimating the concentration of cations adjacent to the polyuronates.

The changes in apoplastic pH and ionic properties in ripening tomato fruit could have significant impact on the catalytic and binding properties of cell wall-associated enzymes. Tomato polygalacturonase, for example, is nearly inactive at the pH values ≥ 6.0 and low ionic strength of the apoplast of mature-green fruit (Chun and Huber, 1998). Although the decline in pH during ripening should enhance the activity of this enzyme, catalysis might still be limited by apoplastic K^+ which, at 37 mM, is about one-third of the level required for maximum PG activity in vitro (Chun and Huber, 1998). A decline in galactose, possibly mediated by β -galactanase (Pressey, 1983; Carey et al., 1995), is one of the early events in the cell walls of ripening fruit. While the optimum pH for tomato β -galactanase against native galactan is 4.5 (Carey et al., 1995), nearly 2 pH units below the measured values for mature-green fruit, the activity range for this enzyme extends to pH values in excess of 6.0 (Pressey, 1983). Of several isozymes of

pectinmethylesterase reported for tomato fruit (Giovane et al., 1994; Warrilow and Jones, 1995), only one exhibited significant activity below pH 6 (Giovane et al., 1994).

It is clear that apoplastic conditions will have differential effects on the activity and/or binding properties of these and other wall-associated enzymes, and may explain why the activities of extractable enzymes do not always correlate well with their action in situ. Changes in apoplast pH and mineral composition may also influence nonenzymic cell wall disassembly. The extent and rate of the changes in apoplastic conditions during ripening could be influenced by a number of factors, including high or low temperature (chilling injury) extremes. Unscheduled or premature changes in apoplastic conditions may disrupt the coordination between hydrolysis and physical disaggregation of structural polymers, facilitating the development of aberrant textural conditions.

Summary

Apoplastic pH and ionic conditions are known to exert strong influence on cell wall metabolism of many plant tissues; however, the nature of the apoplastic environment of ripening fruit has been the subject of relatively few studies. In this report, a pressure-bomb technique was used to extract apoplastic fluid from tomato fruit pericarp at several developmental stages. pH and the levels of K^+ , Na^+ , Ca^{2+} , Mg^{2+} , Cl^- and P were determined and compared with the values for the bulk pericarp and locule tissues. The pH of the apoplastic fluid from pericarp tissue decreased from 6.7 in immature and mature-green fruit to 4.4 in fully ripe fruit. During the same period, the K^+ concentration increased from 13 to 37 mM. The levels of Na^+ and divalent cations did not change whereas the anions P and Cl^- increased in ripe fruit. Ca^{2+} levels remained relatively

constant during ripening at 4 to 5 mM, concentrations that effectively limit pectin solubilization. The electrical conductivity of the apoplastic liquid showed a 3-fold increase during ripening, whereas osmotically active solutes increased 2-fold. Pressure-treated fruit retained the capacity to ripen. The decline in apoplastic pH and increase in ionic strength during tomato fruit ripening may regulate the activity of specific cell wall hydrolases. The potential role of apoplastic changes in fruit ripening and softening is discussed.

CHAPTER 6

LOW TEMPERATURE EFFECTS ON THE APOPLASTIC ENVIRONMENT OF TOMATO PERICARP

Introduction

Chilling injury is a complex syndrome that develops in many plant organs during or following storage at low, non-freezing, temperatures (Lyons, 1973; Nishida and Murata, 1996). The mechanism that leads to chilling injury has not been convincingly described; however, it is clear that membranes and enzyme kinetics are altered as a result of low-temperature exposure (Parkin et al., 1989; Nishida and Murata, 1996).

During ripening, the apoplastic solution of tomato pericarp is acidified and accumulates K^+ ions (Chapter 5). These alterations are relevant to the regulation of cell wall metabolism during ripening. Pectin disassembly in chilling injured tomato fruit differs from that occurring during ripening (Chapters 3 and 4). The differences can be partially attributed to differential patterns of expression of cell wall hydrolases (Jackman et al., 1992). Additionally, the catalytic activity of wall enzymes may be influenced by the apoplastic pH and mineral composition in chilling-injured fruit. Chilling-induced lateral phase transitions (Sharom et al., 1994) are thought to result in increased electrolyte leakage (King and Ludford, 1983; Autio and Bramlage, 1986; Lurie et al., 1994; Sharom et al., 1994) and are likely to change the pH and mineral composition of the apoplastic

solution. Data presented in a previous chapter showed that electrolyte leakage increased in tomato fruit stored at low-temperature, but not in fruit ripening at 15 °C, suggesting that alterations in the apoplastic environment might differ in ripening and chilling-injured fruit (Chapter 4). The objective of this study was to characterize apoplastic pH and mineral composition in low-temperature exposed tomato fruit.

Materials and Methods

Plant material

Tomato (*Lycopersicon esculentum* Mill.) fruit from line 1436 (Calgene, Davis, CA) containing a PG antisense construct and its untransformed control were field-grown in Gainesville, Florida. The fruit were harvested without calyx at the mature-green stage, as determined by size, washed in 2 mM sodium hypochlorite for 2 min, rinsed with water, and air-dried before storage at the indicated temperatures.

Storage conditions

Mature-green fruit were stored in the dark at 5 °C for 2 weeks, after which time the fruit were transferred to 15 °C. The fruit were aerated with hydrated air at flow rates of 20 and 75 mL min⁻¹ kg⁻¹ when stored at 5 and 15 °C, respectively.

Extraction of apoplastic and bulk fluids

Pressure-extracted, apoplastic fluid was obtained as described in Chapter 5. The fruit were maintained at 5 °C during extraction. The exudate from freshly harvested

mature-green fruit was obtained at 0.7 MPa whereas lower pressures were adequate to expel fluid from chilled fruit. Fruit stored at 5 °C for 2 weeks required 0.5 MPa, whereas fruit after 5 days at 15 °C following cold storage required 0.3 to 0.4 MPa. Following fluid extraction, each fruit was cut and the maturity stage confirmed by the status of the locular tissue. Immature fruit were discarded at this stage.

Bulk pericarp sap was obtained from the same fruit used for extraction of apoplastic fluid. Approximately 4 g of frozen pericarp tissue were thawed and centrifuged at 1250g for 20 minutes. The supernatant was collected and used for pH and osmolality determinations. Osmolality was measured in 10 μ L of sample with a Wescor vapor pressure osmometer (model 5500, Logan, UT) calibrated with 290 and 1000 mmol kg^{-1} standards. pH was measured with a Lazar combination microelectrode (model PHR-146, Los Angeles, CA). The concentrations of K^+ , Ca^{2+} , Mg^{2+} were determined by inductively coupled plasma spectroscopy (model ICAP 9000, Thermo Jarrell Ash Corporation, Franklin, MA). Since no differences in the measured variables were observed between the polygalacturonase antisense and wild-type fruit, values are presented averaged over both lines.

Results

Osmolality of apoplastic and bulk fluids

The osmolality of the pressure-extracted fluid was compared with the osmolality of bulk fluid in order to assess the degree of contamination of the samples with exudates of protoplasmic origin or by infiltration of liquid from the locular cavity. Samples with

lowest osmolality were considered representative of the apoplastic solution and used for further analyses.

The average osmolality of the bulk fluid was 271 mOsmol kg⁻¹, remaining constant during storage at 5 °C and following transfer to 15 °C (Figure 6-1). The apoplastic fluid of mature-green fruit had an osmolality of 95 mOsmol kg⁻¹. During the 2-week storage at 5 °C, the osmolality of the apoplastic fluid increased to 120 mOsmol kg⁻¹ and continued increasing at a higher rate upon transfer of the fruit to 15 °C to reach 142 mOsmol kg⁻¹ after 6 days at 15 °C following low-temperature storage (Figure 6-1).

pH of apoplastic and bulk fluids

Freshly harvested mature-green fruit exhibited an apoplastic pH of 5.7, whereas the bulk fluid had a pH of 4.7. Storage at 5 °C caused a drastic reduction in apoplastic pH to 4.5 and a further reduction to 4.3 occurred during subsequent storage at 15 °C to values more acidic than the bulk fluid (Figure 6-2).

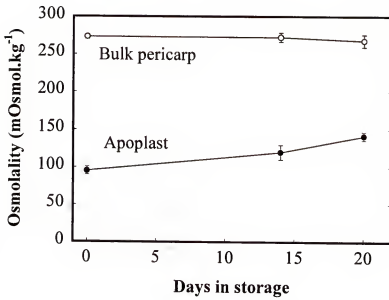


Figure 6-1. Osmolality of the apoplastic (●) and bulk (○) fluids extracted from tomato pericarp stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days. Values are means $\pm$ SE of 6 observations.

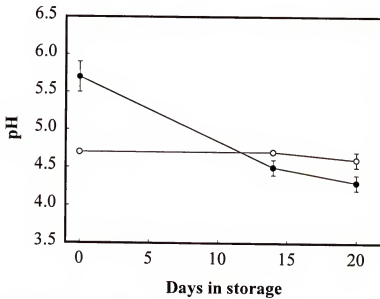


Figure 6-2. pH of the apoplastic (●) and bulk (○) fluids extracted from tomato pericarp stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days. Values are means $\pm$ SE of 6 observations.

Inorganic mineral concentration in apoplastic and bulk fluids

The divalent cations Ca^{2+} and Mg^{2+} were more abundant in the apoplastic fluid (Figure 6-3 and 6-4), whereas K^+ was more abundant in the bulk fluid (Figure 6-5). The apoplastic fluid of mature-green fruit at harvest had 5.9 mM Ca^{2+} and 18.1 mM Mg^{2+} , whereas the concentrations in the bulk fluid were 0.2 mM Ca^{2+} and 2.6 mM Mg^{2+} . The concentrations of both divalent cations in the apoplastic fluid remained constant during low-temperature storage, but declined by 45% after 6 days at 15 °C following the low-temperature storage (Figures 6-3 and 6-4). The levels of divalent cations in the bulk fluid were not affected by storage at 5 °C or during storage at 15 °C.

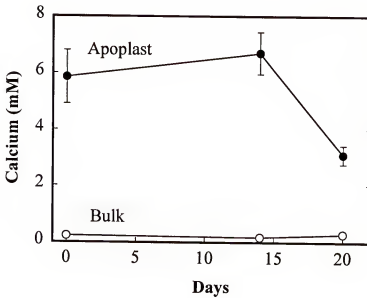


Figure 6-3. Concentration of calcium in the apoplastic (●) and bulk (○) fluids extracted from tomato pericarp stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days. Values are means $\pm$ SE of 6 observations.

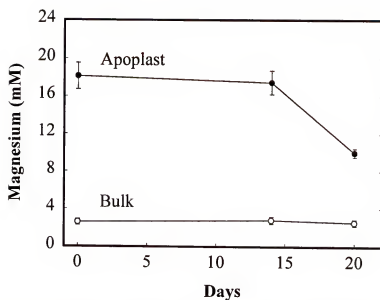


Figure 6-4. Concentration of magnesium in the apoplastic (●) and bulk (○) fluids extracted from tomato pericarp stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days. Values are means $\pm$ SE of 6 observations.

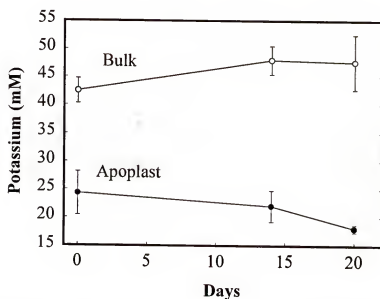


Figure 6-5. Concentration of potassium in the apoplastic (●) and bulk (○) fluids extracted from tomato pericarp stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days. Values are means $\pm$ SE of 6 observations.

The most abundant inorganic ion in the apoplastic fluid of mature-green fruit was K^+ (24.3 mM). K^+ levels remained unaltered during storage at 5 °C and decreased slightly when the fruit were removed to 15°C. Its concentration in the bulk fluid increased from 42.5 to 47.9 mM during low-temperature storage and remained nearly unchanged during storage at 15 °C (Figure 6-5).

Discussion

Details on the effects of low-temperature storage on color, firmness, electrolyte leakage, and pectin metabolism were presented in Chapter 4. Briefly, during storage of mature-green fruit at 5 °C for 14 days there was a slight yellowing of the fruit surface. Subsequent transfer of the fruit to 15 °C resulted in uneven development of red hue, indicating mild chilling injury. A slight decrease in pericarp firmness occurred during low-temperature storage, and subsequent transfer to 15 °C did not result in further softening. Pectin solubilization and molecular mass distribution in chilling injured fruit were similar to those of mature-green fruit prior to storage at 5 °C, indicating that ripening-related alterations in the pectic matrix had not occurred. Electrolyte leakage, expressed as a percentage of total tissue electrolytes, remained constant or decreased during storage at 5 °C in wild-type and PG-antisense fruit, respectively. Electrolyte leakage decreased upon transfer of chilled fruit to 15 °C and during subsequent storage at 15 °C in both wild-type and PG-antisense fruit.

The physicochemical properties of membranes change as a result of low-temperature storage (Parkin et al., 1989). Low-temperature storage can result in an increase in electrolyte leakage (expressed as percentage of total) from tomato pericarp

(Bergevin et al., 1993; Autio and Bramlage, 1986; King and Ludford, 1983; Sharom et al., 1994). Upon transfer of fruit to non-chilling temperatures following cold storage, electrolyte leakage has also been reported to increase (Sharom et al., 1994) or decrease (Bergevin et al., 1993) in comparison with the level at the end of cold storage. Increased electrolyte leakage, expressed as a percent of total electrolytes, is often interpreted as evidence for damage to the plasma membrane. Indeed, x-ray diffraction studies provide evidence that membranes undergo liquid-crystalline to solid-gel phase transitions upon transfer from cold storage to non-chilling temperatures (Sharom et al., 1994). However, electrolyte leakage presents limitations in assessing membrane permeability since it does not distinguish between membrane competence and confounding variables that may affect ion flux (e.g. relative water content of the tissue, Whitlow et al., 1992). Revised protocols based on the law of diffusion have been proposed to better relate the conductivity measurements with membrane leakiness (Whitlow et al., 1992), although in many instances the revised protocols yield results similar to those obtained by traditional methods (Manley and Hummel, 1996).

Gross changes in membrane permeability during low-temperature storage would facilitate the equilibration of pH and solutes between the cytosol and the apoplastic solution, dramatically altering the apoplastic environment. However, major permeability changes do not seem to occur in response to chilling. The osmolality of the pressure-extracted fluid from freshly harvested mature-green fruit was just 35% of that of the bulk fluid, similar to the values reported for immature fruit although slightly higher than the osmolality of mature-green fruit observed in a previous experiment (Chapter 5). Ruan et al. (1995) reported an osmolality of the apoplastic fluid of immature fruit 48% lower than

the bulk fluid. The osmolality of putative apoplastic fluid remained at 40% and 50% of that of the bulk fluid after 2 weeks at 5 °C and following 6 days at 15 °C, respectively, indicating minimal compromise in compartmentation. The minimal change in the osmolality of the apoplastic fluid during low-temperature storage is in agreement with evidence that membrane properties remain essentially unaltered during cold storage (Sharom et al., 1994). The increase in total solutes in the apoplast when fruit were returned to higher temperature after cold storage was small. Even in chill-injured fruit, the apoplast had only about half the total solute concentration as the bulk solution, indicating that tissue compartmentation was not seriously compromised. It is unknown whether more severe injury might yield different results.

The apoplastic fluid of freshly harvested mature-green fruit had a pH of 5.7, lower than the value of 6.7 found in a previous study of immature and mature-green tomato (Chapter 5), but close to the value 6.0 reported for immature fruit by Ruan et al. (1995). During storage of tomato fruit at 5 °C, the apoplastic pH decreased to 4.5. After 6 days at 15 °C following storage at 5 °C the apoplastic fluid had a pH of 4.3. The latter value is similar to that of the apoplastic fluid of ripe fruit (Chapter 5), although chilling injured fruit did not ripen (Chapter 4). The acidification of the apoplast, despite small variation in osmolality, may be caused by plasma membrane ATPase, whose activity increases in response to freezing injury (Iswari and Palta, 1989), and may also be enhanced during exposure to chilling temperatures. The bulk pH of freshly harvested mature-green fruit (4.7) was slightly higher than values (4.5) reported for mature-green fruit in Chapter 5 and remained constant during storage at 5 °C. In tomato fruit, low-temperature storage induced inconsistent changes in bulk pH (Maul, 1999), but in fruit of *Annona* species,

low-temperature storage (1-4 °C) inhibited the ripening-related decline in mesocarp bulk pH (Gutiérrez et al., 1994).

The levels of soluble apoplastic cations remained constant during low-temperature storage and declined after transfer to 15 °C. This is in contrast to the reported increase in K⁺ efflux from pepper pericarp disks incubated in 350 mM sorbitol (Lurie et al., 1994) and the increased efflux of K⁺, Na⁺ and Mg²⁺ from sweet potato tissue incubated in water, as a result of cold storage (Murata and Tatsumi, 1979).

The regulation of apoplastic mineral composition in chilling-injured fruit may be complex. Saltveit (1989) has proposed a dual compartment model for the interpretation of efflux data, where the apoplast would be a fast-release compartment unaffected by chilling, and the protoplast a slow-release compartment whose leakiness would be affected by chilling. The model neglects the possibility of chilling-induced changes in the density of negative charges in the cell wall (Côté et al., 1993). The levels of apoplastic cations remained constant or increased during ripening (Chapter 5), whereas decreased levels were consistently observed after transfer of fruit stored at 5 °C to 15 °C. It is not clear whether electrostatic conditions of the apoplast can account for the different patterns of accumulation of soluble cations in ripening and chilling-injured fruit. Although extractable pectinmethylesterase has been reported to increase during cold storage (Fukushima, 1978; Marangoni et al., 1995) the rate of deesterification is reduced in fruit stored at 5 °C compared with those allowed to continue normal ripening (Jackman et al., 1992). Moreover, Jackman et al. (1992) reported an increase in pectin methylesterification when fruit were transferred to 22 °C following storage at 5 °C for 15 days. The slight increase in osmolality after low-temperature storage coincided with a

decrease in the levels of the major soluble cations. This increase in osmolality might be accounted for by efflux of sugars and organic acids.

Conductance (electrolyte leakage) measurements do not totally reflect the stress-induced changes in membrane permeability. Conductivity correlates closely with K^+ efflux, but this cation and corresponding counterion accounted for only 20% of the total solutes leaked from freezing-injured cells (onion scales), the remaining 80% being sugars (Palta et al., 1977). Rather than increasing gross membrane permeability, low-temperature storage may cause changes in selective permeability induced by altered behavior of proton pumps and ion channels. For example, mild, reversible freezing injury induces an increase in the specific activity of the plasma membrane ATPase (Iswari and Palta, 1989). Similar changes may occur in other ion-channels.

Chilling injury induced changes in the apoplastic pH and mineral composition, possibly by affecting both the permeability of the plasma membrane and the buffering capacity of the cell wall. A decrease in the solubility of apoplastic minerals following low-temperature storage would obscure the effects of temperature on membrane permeability. Collectively, the data reported in this study do not support the idea that severe membrane dysfunction occurs during or following exposure of tomato fruit to low temperatures.

Summary

Low-temperature storage of chilling sensitive commodities affects the function of membranes and is likely to induce alterations in the apoplastic environment. In this study, apoplastic osmolality, pH, K^+ , Ca^{2+} , and Mg^{2+} levels were examined in mature-green

tomato fruit stored at 5 °C for 14 days and subsequently stored at 15 °C for an additional 6 days. The osmolality of the apoplastic fluid was 35% of that of the bulk tissue before storage, increased slightly during storage at 5 °C to 40 % of the bulk osmolality and to 50% of bulk osmolality after transfer of chilled fruit to 15 °C for 6 days. Storage of mature-green tomato at 5 °C for 14 days induced a decrease in apoplastic pH from 5.7 in freshly harvested mature-green fruit to 4.5 after storage at 5 °C, and a further decrease to 4.3 following transfer to 15 °C for 6 days. Apoplastic minerals remained at constant levels during low-temperature storage at about 6, 18, and 24 mM for Ca^{2+} , Mg^{2+} , and K^{+} , respectively. Storage at 15 °C following low-temperature storage resulted in a 45% decrease in apoplastic Ca^{2+} and Mg^{2+} , and a 20% decrease in soluble apoplastic K^{+} . The decreases in soluble apoplastic cations after removal of the fruit from low-temperature storage are consistent with reported decreases in electrolyte leakage. This study provides evidence against the severe membrane dysfunction in chill-injured fruit.

CHAPTER 7

IN VIVO SOLUBILIZATION OF PECTINS, NEUTRAL SUGARS, AND FREE GALACTOSE IN RIPENING AND CHILL-INJURED TOMATO FRUIT

Introduction

Plant cells are encapsulated by a wall, a complex entity composed of polysaccharides, structural proteins, and enzymes. In dicots, pectic polysaccharides account for about one-third of the cell wall material (Darvill et al., 1980). Pectin metabolism plays critical roles in the development of plant organs. Pectins determine apoplastic porosity (Baron-Epel et al., 1988), cell adherence (Knee, 1978), and ion-exchange capacity (Gillet et al., 1998). Some pectic oligosaccharides have signaling properties, the best characterized of which are elicitor and morphogenetic effects (Fry et al., 1993).

In fleshy fruits, including tomato, ripening is accompanied by cell wall disassembly, a complex process involving both enzymic and nonenzymic mechanisms. The pectic network is particularly targeted during ripening, undergoing deglycosylation, deesterification, solubilization, and depolymerization (Huber, 1983) although synthesis persists (Knee, 1982; Greve and Labavitch, 1991; Huysamer et al., 1997).

Evidence has shown that some of the enzymes implicated in the disassembly of the pectic network are catalytically restricted *in vivo*. The potential activity of PG (EC 3.2.1.15) *in vitro* exceeds the level necessary to explain the compositional changes found

in fruit cell walls (Seymour et al., 1987). The ripening-related changes in pectic polymers have been ascertained from analysis of isolated cell wall polysaccharides. The isolation procedures involve tissue homogenization, organic solvent precipitation of polysaccharides, and various washing procedures. The classic pectic fractions have been categorized on the basis of solubility in water, chelators and alkalis; however, how these solubility fractions reflect changes in pectin properties *in vivo* is unknown. Previous studies addressed pectin solubilization from isolated ethanol insoluble solids (EIS) (Chapter 3) and from pericarp disks incubated in isotonic media (Chapter 4). The ripening-related increase in pectin efflux from pericarp disks was largely PG-independent and much lower than the PG-dependent release of water-soluble pectins from EIS. These results suggest that *in vivo* pectin solubilization is restricted and PG-independent.

In Chapter 5, apoplastic pH and mineral composition in ripening tomato fruit were characterized by extracting and analyzing a fluid from apoplastic origin. This study revealed that the apoplast contains about 5 mM of Ca^{2+} throughout ripening (Chapter 5), a concentration that is sufficient to strongly limit enzymic pectin solubilization from isolated cell walls (Rushing and Huber, 1987). It is likely, however, that a portion of the apoplastic Ca^{2+} is removed during cell wall isolation, chelated by organic acids during tissue homogenization. Moreover, the use of phenol-acetic acid-water (2:1:1, w/v/v) to inactivate endogenous enzymes displaces as much as 50% of cell wall Ca^{2+} levels, significantly altering pectin solubility in wall isolates (Huber, 1991). Given the critical role of calcium in determining pectin integration in the cell wall (Jarvis, 1982), pectin solubility from isolated cell walls likely does not reflect solubility in the apoplast.

The objective of this study was to characterize *in vivo* pectin solubilization during ripening and as a result of low-temperature storage, by determining the levels of polyuronides in a pressure-extracted apoplastic fluid. Fruit with reduced levels of PG were used to further investigate the influence of PG on polyuronide solubilization.

Materials and Methods

Plant material and storage regimes

Tomato (*Lycopersicon esculentum* Mill.) fruits from line 1436 (Calgene, Davis, CA) containing a PG antisense construct and corresponding nontransformed wild-type were harvested at the mature-green stage from field-grown plants, washed in 2 mM sodium hypochlorite for 2 min, rinsed and air-dried. Fruit were stored at 15 °C and sampled at the mature-green, pink, and ripe stages. Other fruit were stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days.

Extraction of apoplastic and bulk fluids

Apoplastic fluid was extracted with an adaptation of the pressure bomb procedure used by Ruan et al. (1995) as previously described (Chapter 5). The extractions were performed at 5 °C using a pressure of 0.7 MPa for mature-green and 0.4 MPa for ripe fruit. Fruit stored at 5 °C for 2 weeks were pressurized at 0.5 MPa for fluid extraction whereas 0.3-0.4 MPa were required to expel fluid from fruit transferred to 15 °C for 6 days after cold storage. An aliquot of 100 µL of pressure-extracted fluid was added to 400 µL of ethanol and stored at -20 °C until analyzed.

Bulk pericarp sap was extracted from the same fruit used for apoplastic fluid extraction. Approximately 4 g of frozen pericarp tissue were thawed at room temperature and centrifuged at 1250g for 20 minutes. The supernatant was collected and used for analyses.

Extraction of proteins and polysaccharides from apoplastic and bulk fluids

Apoplastic and bulk fluid isolates in 80 % ethanol were heated at approximately 90 °C in a water bath for 15 minutes in capped polypropylene vials and stored 12 h at – 20 °C. The samples were centrifuged for 10 minutes at 8500g (Biofuge 15, Heraeus Instruments, South Plainfield, NJ) and the supernatant collected for analysis. The pellets were resuspended in 0.5 mL of 80 % ethanol, centrifuged for 10 minutes at 8500g and the supernatant discarded. The pellet was then suspended in 1.0 mL of distilled water for subsequent analyses.

Total protein and uronic acid determination

Total protein in apoplastic and bulk fluids was determined by the bicinchoninic acid method (Smith et al., 1985). Two mL of a mixture of bicinchoninic acid solution (Sigma) and 4 % (w/v) cupric sulfate in the proportion of 50:1 were added to 0.1 mL of sample and incubated at 60 °C for 30 minutes in a water bath. After cooling to room temperature the absorbance was determined at 562 nm with a Beckman spectrophotometer (DU 640, Fullerton, CA). Bovine serum albumin (Sigma) was used as a standard. Total uronic acids were determined using the *m*-hydrodiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) with galacturonic acid as a standard.

Free galactose

The 80% ethanolic supernatants of the apoplastic and bulk fluids were evaporated to dryness under a stream of air in a heating block at 38 °C. Residues were suspended in 1.0 mL of distilled water. Galactose levels were determined using a galactose oxidase/peroxidase assay adapted from Sturgeon (1994). Briefly, 0.1 mL of sample was incubated for 3 h at 37 °C with 2.9 mL of a solution of 20 units of D-galactose oxidase (EC 1.1.3.9, Sigma), 1250 units of horseradish peroxidase (EC 1.11.1.7, Sigma), and 5 mg of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) per 100 mL of 100 mM phosphate buffer, pH 7.0. At the end of the incubation period, the absorbance was measured at 440 nm. D-galactose was used as a standard.

Neutral sugars in the apoplastic soluble polymers

Ethanol-insoluble pellets suspended in distilled water were dried at 35 °C under a stream of filtered air. Hydrolysis and derivatization procedures were as described by Albersheim et al. (1967) with minor modifications. The neutral sugar-containing polysaccharides were hydrolyzed in 2 N trifluoroacetic acid at 120 °C for 1 h. The resulting monosaccharides were reduced with 0.66 M sodium borohydride in 1 N ammonium hydroxide overnight at 25 °C. The samples were acidified with Dowex 50W until the pH was around 5. The resin was removed by filtration through a syringe-mounted GF/C filter paper and the samples were dried and subsequently washed 3 times with methanol and once with ethanol before derivatization. The sugars were converted into acetyl derivatives in the presence of 0.2 mL of acetic anhydride and 0.2 mL of

pyridine for 1 h at 100 °C. The samples were then dried, washed with toluene 3 times, and solubilized in methylene chloride. The sugars were separated on a 25 m crosslinked 5% phenyl substituted methylsiloxane capillary column (HP-5, 0.2 mm internal diameter, 0.33 μm film thickness) using a 5890 series II HP gas-chromatograph equipped with a flame ionization detector. Helium was used as the carrier gas at 60 psi. The oven was held at 210 °C for 5 min, increased to 230 °C at 4 °/min, and held at this temperature for 14 min. The quantification of individual sugars was accomplished using *myo*-inositol as an internal standard.

Results

Soluble protein in the apoplastic and bulk fluids

Apoplastic solutions contained protein at all stages of ripening, ranging from 224 to 475 $\mu\text{g mL}^{-1}$ (Table 7-1). The concentration of protein was highly variable, decreasing from the mature-green to the pink stage and then increasing in ripe fruit. There was an overall decrease (18%) in soluble apoplastic protein in PG-antisense fruit and an increase (30%) in wild-type. Protein was 20 times more dilute in bulk than in apoplastic fluids at the mature-green stage. As the fruit ripened, the concentration of soluble protein in the bulk fluid increased 3-fold in PG-antisense fruit and 27-fold in wild-type fruit. At the ripe stage, wild-type fruit yielded 10 times more soluble protein in the bulk fluid than the PG-antisense fruit (Table 7-1).

Table 7-1. Protein concentration in the apoplastic and bulk fluids of tomato pericarp extracted from PG-antisense (AS) and wild-type (WT) fruit at several ripening stages. Values are means $\pm$ SE of 4 observations.

Stage	Apoplast ($\mu\text{g mL}^{-1}$)		Bulk ($\mu\text{g mL}^{-1}$)	
	AS	WT	AS	WT
MG	403 $\pm$ 84	363 $\pm$ 124	17 $\pm$ 5	20 $\pm$ 10
Pink	273 $\pm$ 52	224 $\pm$ 42	59 $\pm$ 15	302 $\pm$ 43
Red	331 $\pm$ 87	475 $\pm$ 192	53 $\pm$ 8	537 $\pm$ 225

Table 7-2. Protein concentration in the apoplastic and bulk fluids of tomato pericarp extracted from PG-antisense (AS) and wild-type (WT) fruit stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days. Values are means $\pm$ SE of 4 observations.

Stage	Apoplast ($\mu\text{g mL}^{-1}$)		Bulk ($\mu\text{g mL}^{-1}$)	
	AS	WT	AS	WT
Before storage	403 $\pm$ 84	363 $\pm$ 124	17 $\pm$ 5	20 $\pm$ 10
14 d at 5 °C	308 $\pm$ 43	272 $\pm$ 36	63 $\pm$ 11	72 $\pm$ 13
14 d at 5 °C + 6 d at 15 °C	311 $\pm$ 44	425 $\pm$ 75	202 $\pm$ 16	427 $\pm$ 266

The concentration of protein recovered from the apoplastic fluid decreased during storage of mature-green fruit at 5 °C for 14 days (Table 7-2). After 6 days at 15 °C following cold storage, protein concentration remained constant at about 310 $\mu\text{g mL}^{-1}$ in PG-antisense fruit but increased more than 50% in wild-type fruit (Table 7-2). Soluble protein in the bulk fluid increased 3.6 fold during storage of the fruit at 5 °C, with no effect of PG levels. Following transfer of fruit to 15 °C for 6 days, protein concentration

in the bulk fluid was strongly affected by PG levels, with the wild-type yielding twice the amount of protein released by PG-antisense bulk fluid (Table 7-2).

Total ethanol-insoluble pectins in the apoplastic and bulk solutions

The concentration of pectins recovered in the apoplastic fluid increased 3-fold during ripening from $220 \mu\text{g mL}^{-1}$ at the mature-green stage to about $680 \mu\text{g mL}^{-1}$ at the ripe stage (Table 7-3), with no effect of PG levels. In contrast with the apoplastic fluid, PG levels strongly affected pectin concentration in bulk fluids (Table 7-3). Soluble pectins increased 14-fold during ripening of PG-antisense fruit whereas a 36-fold increase was observed in the wild-type.

The concentration of pectins in the apoplastic fluid decreased slightly during cold storage, but increased considerably upon transfer to 15°C in a PG-independent manner (Table 7-4). In the bulk fluid, pectin concentration changed little during low-temperature storage but solubilization increased dramatically after transfer to 15°C for 6 days (Table 7-4).

Table 7-3. Pectin concentration in the apoplastic and bulk fluids of tomato pericarp extracted from PG-antisense (AS) and wild-type (WT) fruit at several ripening stages. Values are means $\pm$ SE of 4 observations.

Stage	Apoplast ($\mu\text{g mL}^{-1}$)		Bulk ($\mu\text{g mL}^{-1}$)	
	AS	WT	AS	WT
MG	230 $\pm$ 38	208 $\pm$ 61	19 $\pm$ 7	31 $\pm$ 19
Pink	433 $\pm$ 99	176 $\pm$ 82	169 $\pm$ 34	814 $\pm$ 60
Red	599 $\pm$ 157	764 $\pm$ 403	261 $\pm$ 18	1127 $\pm$ 286

Table 7-4. Pectin concentration in the apoplastic and bulk fluids extracted from tomato pericarp extracted from PG-antisense (AS) and wild-type (WT) stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days. Values are means $\pm$ SE of 4 observations.

Stage	Apoplast ($\mu\text{g mL}^{-1}$)		Bulk ($\mu\text{g mL}^{-1}$)	
	AS	WT	AS	WT
Before storage	230 $\pm$ 38	208 $\pm$ 61	19 $\pm$ 7	31 $\pm$ 19
14 d at 5 °C	136 $\pm$ 15	122 $\pm$ 14	25 $\pm$ 10	21 $\pm$ 13
14 d at 5 °C + 6 d at 15 °C	333 $\pm$ 68	274 $\pm$ 19	347 $\pm$ 122	483 $\pm$ 185

Galactose levels in apoplastic and bulk solutions

The concentration of free galactose in the bulk solution increased from 163 and 159 $\mu\text{g mL}^{-1}$ at the mature-green stage in antisense and wild-type fruits, respectively, to 192 $\mu\text{g mL}^{-1}$ at the ripe stage in both lines (Table 7-5). Galactose concentrations in the apoplast were lower and increased during ripening from 44 to 70 $\mu\text{g mL}^{-1}$ in antisense fruit and from 39 to 60 $\mu\text{g mL}^{-1}$ in wild-type fruits.

Storage of mature-green fruit at 5 °C for 14 days resulted in 50% lower bulk free-galactose concentration compared with concentrations in pre-chilling fruit (Table 7-6). Following transfer of chilled fruit to 15 °C, additional changes in bulk free galactose were not evident. Apoplastic free galactose concentrations were much lower than bulk values and decreased from an average of 41 $\mu\text{g mL}^{-1}$ in freshly harvested mature-green fruit to

34 $\mu\text{g mL}^{-1}$ during storage at 5 °C and remained unchanged during subsequent storage at 15 °C.

Table 7-5. Concentration of free galactose in the apoplastic and bulk fluids extracted from ripening tomato pericarp. Values are means $\pm$ SE of 4 observations.

Stage	Apoplast ($\mu\text{g mL}^{-1}$)		Bulk ($\mu\text{g mL}^{-1}$)	
	AS	WT	AS	WT
MG	44 $\pm$ 8	39 $\pm$ 4	163 $\pm$ 12	159 $\pm$ 2
Pink	53 $\pm$ 6	53 $\pm$ 16	168 $\pm$ 5	168 $\pm$ 1
Red	70 $\pm$ 6	60 $\pm$ 17	192 $\pm$ 6	192 $\pm$ 14

Table 7-6. Concentration of free galactose in the apoplastic and bulk fluids extracted from mature-green tomato pericarp before and after 14 days of storage at 5 °C, and after 6 days at 15 °C following storage at 5 °C. Values are means $\pm$ SE of 4 observations.

Stage	Apoplast ($\mu\text{g mL}^{-1}$)		Bulk ($\mu\text{g mL}^{-1}$)	
	AS	WT	AS	WT
Before storage	44 $\pm$ 8	39 $\pm$ 4	163 $\pm$ 12	159 $\pm$ 2
14 d at 5 °C	33 $\pm$ 4	34 $\pm$ 7	80 $\pm$ 4	77 $\pm$ 8
14 d at 5 °C + 6 d at 15 °C	30 $\pm$ 1	38 $\pm$ 4	82 $\pm$ 10	80 $\pm$ 6

Polymeric neutral sugars in the apoplastic fluid

Xylose and galactose were the most abundant noncellulosic neutral sugars in the ethanol-insoluble fraction of the apoplastic fluid of ripening fruit, followed by glucose and arabinose (Table 7-7). The proportion of rhamnose and arabinose tended to increase during ripening, whereas xylose, mannose and glucose decreased.

Low-temperature storage induced significant reductions in the levels of rhamnose and glucose, and increases in the proportion of xylose (Table 7-8). After transfer of fruit to 15 °C for 6 days following cold storage, the proportion of xylose increased whereas arabinose, galactose, and mannose decreased.

Table 7-7. Neutral sugars in the ethanol-insoluble polymers from the apoplastic fluid of ripening tomato (expressed as a mol percentage of total galacturonic acid). Values are means $\pm$ SE of 2 observations.

Line	Stage	Rha	Ara	Xyl	Man	Glc	Gal
		mol %					
WT	MG	2.1 $\pm$ 1.3	17.2 $\pm$ 5.1	33.1 $\pm$ 18.8	7.3 $\pm$ 1.2	14.9 $\pm$ 5.0	25.4 $\pm$ 8.8
WT	Pink	10.0 $\pm$ 5.2	16.8 $\pm$ 3.5	33.7 $\pm$ 14.2	4.8 $\pm$ 1.5	15.1 $\pm$ 1.8	19.7 $\pm$ 2.3
WT	Ripe	4.6 $\pm$ 1.0	22.2 $\pm$ 8.2	27.3 $\pm$ 18.3	5.7 $\pm$ 1.8	17.2 $\pm$ 5.2	23.0 $\pm$ 4.1
AS	MG	3.1 $\pm$ 2.0	16.9 $\pm$ 1.9	24.0 $\pm$ 4.6	11.0 $\pm$ 1.1	20.9 $\pm$ 1.0	24.1 $\pm$ 2.7
AS	Pink	9.8 $\pm$ 3.1	25.9 $\pm$ 0.5	11.8 $\pm$ 1.3	4.0 $\pm$ 0.3	21.4 $\pm$ 0.6	27.2 $\pm$ 1.7
AS	Ripe	11.9 $\pm$ 2.4	25.3 $\pm$ 0.9	7.9 $\pm$ 0.6	4.6 $\pm$ 1.4	16.9 $\pm$ 3.0	33.4 $\pm$ 3.7

Table 7-8. Neutral sugars in the ethanol-insoluble polymers from the apoplastic fluid of tomato fruit stored at 5 °C for 2 weeks and subsequently transferred to 15 °C for 6 days (expressed as a mol percentage of total galacturonic acid). Values are means $\pm$ SE of 2 observations.

Line	Sampling date	Rha	Ara	Xyl	Man	Glc	Gal
		mol %					
WT	Before storage	2.1 $\pm$ 1.3	17.2 $\pm$ 5.1	33.1 $\pm$ 18.8	7.3 $\pm$ 1.2	14.9 $\pm$ 5.0	25.4 $\pm$ 8.8
WT	14 d at 5 °C	ND	14.7 $\pm$ 4.1	49.6 $\pm$ 6.0	6.2 $\pm$ 0.4	10.2 $\pm$ 1.4	19.4 $\pm$ 3.8
WT	14 d at 5 °C + 6 d at 15 °C	ND	10.5 $\pm$ 0.2	72.0 $\pm$ 0.5	ND	6.1 $\pm$ 0.7	11.4 $\pm$ 0.1
AS	Before storage	3.1 $\pm$ 2.0	16.9 $\pm$ 1.9	24.0 $\pm$ 4.6	11.0 $\pm$ 1.1	20.9 $\pm$ 1.0	24.1 $\pm$ 2.7
AS	14 d at 5 °C	ND	13.9 $\pm$ 2.5	48.2 $\pm$ 0.4	7.2 $\pm$ 1.7	13.9 $\pm$ 2.9	16.8 $\pm$ 1.8
AS	14 d at 5 °C + 6 d at 15 °C	ND	9.2 $\pm$ 1.1	71.3 $\pm$ 1.4	3.1*	7.0 $\pm$ 1.1	10.9 $\pm$ 0.9

* Detected in a single sample; ND- not detected

Discussion

The data reported in this study support the idea that the solubilization of pectins and associated polymers in the fruit apoplast differ from solubilization from isolated cell walls. The presence of about 5 mM of Ca²⁺ in the fruit apoplast, and the possibility for Ca²⁺ removal by cell wall isolation protocols, suggest that in vivo conditions for pectin solubilization and depolymerization may be less than optimal. To assess in vivo pectin solubility, apoplastic fluid from ripening and chill-injured tomato fruit was extracted and analyzed.

Apoplastic protein concentration increased moderately (30%) during ripening of wild-type fruit but decreased in PG-antisense fruit. In the bulk fluids however, there were dramatic increases in protein concentration between the mature-green and ripe stages. Bulk levels of proteins were about 5% of apoplastic levels at the mature-green stage and remained at 16% of apoplastic levels in ripe PG-antisense fruit but increased above the apoplastic levels in wild-type fruit. PG is a major protein in tomato fruit, accumulating in the pericarp at the onset of ripening and increasing to $40\text{--}50\ \mu\text{g g}^{-1}$ f.w. at the ripe stage (Tucker and Grierson 1982). The small difference in apoplastic protein levels between PG-antisense and wild-type fruit suggests that PG is largely bound to the cell wall, in agreement with the results obtained *in vitro* by Rushing and Huber (1990). Tissue disruption occurring in response to freezing and thawing might create conditions similar to those in fruit homogenates (Brummell and Labavitch, 1997). Increased ionic strength caused by disruption of the tissue may release bound PG, explaining the large difference between bulk protein concentration in PG-antisense and wild-type fruit.

The role of PG in *in vivo* pectin solubilization is not clear. In tomato fruit, water solubility of polyuronides correlates with PG levels (Carrington et al. 1993, see also Chapter 3). However, a 99% reduction of PG activity by antisense did not affect the chelator-solubility of pectins (Smith et al. 1990), whereas the expression of PG in the *rin* mutant strongly enhanced the chelator-solubility of polyuronides (Giovannoni et al. 1989). The results presented here indicate that *in vivo* solubilization of pectin in ripening tomato pericarp is largely PG-independent. The limited *in vivo* pectin solubilization may be explained by the high levels of divalent cations present in the apoplast throughout ripening (Chapter 5).

In contrast with the solubilization in the apoplastic fluid of wild-type and PG-antisense fruit, pectins recovered in bulk tissue isolates differed significantly between the two lines. The acceleration of pectin degradation upon tomato tissue disruption, first observed by Kertesz (1938), has been shown in a number of studies (Brummell and Labavitch, 1997; Errington et al., 1998). Although the basis for increased activity is unknown, the sensitivity of PG to low pH and elevated monovalent cation levels (Chun and Huber, 1997, 1998), conditions created upon tissue disruption (Chapter 5), may be involved. Moreover, tissue disruption may reduce the concentration of divalent cations available for cross-linking pectic polymers. This might occur by dilution of the apoplastic solution with symplastic fluid, or by chelation of apoplastic calcium by organic acids released during breakdown of tissue compartmentation.

Low-temperature storage induced a reduction in pectin solubilization in the apoplastic fluid. Transfer of the fruit to 15 °C following 14 days of storage at 5 °C resulted in more than 2-fold increase in apoplastic pectin solubilization in PG-antisense and wild-type fruit. Kukushima and Yamazaki (1978) reported that low-temperature storage (0 °C) decreased hot-water-soluble pectins in chilling sensitive plant organs (banana and cucumber fruits, and sweet potato roots), but not in chilling tolerant green peas and turnip roots. These results support the previous observation that pectin metabolism in chilled fruit differs from that occurring during normal ripening (Chapter 3 and 4).

Galactose loss is a nearly ubiquitous feature of cell wall changes in ripening fruit and originates from pectins or hemicelluloses. Infiltration of galactose into mature-green fruit accelerates the onset of ripening, suggesting that monomeric galactose has

morphogenetic effects in plants (Kim et al., 1987). Several studies have shown that the decrease in cell wall galactosyl residues is accompanied by an increase in free galactose in bulk fluid (Gross and Saltveit, 1982; Gross, 1983; Gross, 1984; Kim et al., 1991). The results presented here, however, show no correlation between the levels of free galactose in the apoplast and the proportion of galactosyl residues in the polysaccharides recovered in apoplastic fluid. The 63% increase in apoplastic free galactose during ripening (Table 7-5) occurred in parallel with no change or an increase in the proportion of galactosyl residues in soluble apoplastic polysaccharides (Table 7-7). During cold storage, a 20% decrease in free galactose (Table 7-6) was accompanied by a 55% reduction in galactosyl residues in the apoplastic soluble polysaccharides (Table 7-8). Possibly, removal of galactosyl residues does not increase the overall solubilization of polysaccharides under apoplastic conditions, or free galactose originates from polymers distinct from those recovered in the apoplastic exudates.

The regulation of galactose metabolism in ripening fruit remains elusive. Previous determinations of free galactose in ripening tomato used bulk fluids extracted after tissue homogenization. This methodology results in the blend of different pools of galactose, mainly derived from the cell wall and galactolipids. When enzymes were inactivated by refluxing ethanol, bulk pericarp fluids showed a 4-8 fold increase in free galactose during ripening (Gross and Saltveit 1982, Gross 1983). Using enzymically active homogenates, we observed a 20% increase in bulk free galactose between the mature-green and ripe stages. The amount of free galactose in the bulk pericarp of ripe fruit ($192 \mu\text{g mL}^{-1} = 1.07 \text{ mM}$) is within the range reported elsewhere; however, our mature-green samples contained 4-8 times more ($161 \mu\text{g mL}^{-1} = 0.89 \text{ mM}$) galactose than reported for

enzymically inactive fluids (Gross and Saltveit, 1982; Gross, 1983). This possibly indicates that tissue disruption increases the activity of galactosidase at the mature-green stage, possibly due to the apoplastic pH.

The net loss of galactose from cell walls during ripening has been attributed to reduced galactan synthesis (Lackey et al., 1980), or increased hydrolysis by β -galactosidase II (Pressey, 1983; Kim et al., 1991). The increase in free galactose may be a direct result of hydrolysis (Kim et al., 1991), possibly accompanied by a reduced ability to metabolize free galactose (Gross and Saltveit, 1982). β -Galactosidase II, a cell wall β -galactanase in tomato fruit, shows a 3-fold increase in activity during ripening (Pressey, 1983). The enzyme is active between pH 3 and 6.5, with optimum activity around 4.2 and shows a 50% inhibition in the presence of 2 mM galactose (Pressey, 1983). As tomato fruit ripen, the apoplastic pH becomes more favorable for β -galactanase activity (Chapter 5), and the levels of apoplastic galactose at the ripe stage (0.4 mM) are not likely to strongly inhibit the enzyme.

Net losses of cell wall arabinose and galactose are observed during tomato ripening (Wallner and Bloom, 1977; Gross, 1986; Redgwell et al., 1997). An increase in the proportion of arabinosyl residues in the polysaccharides soluble in the apoplast during ripening indicates that loss of arabinose occurs in part due to solubilization of an arabinose-containing polymer instead of the monomeric sugar. Dick and Labavitch (1989) reached a similar conclusion for ripening pear. Carrington et al. (1993) reported a decrease in galactose in Na_2CO_3 -soluble pectins during ripening of PG-antisense and wild-type tomato fruit, but the concomitant increase in galactose in water-soluble pectins

was observed only in wild-type fruit. Moreover, an increase in arabinose levels in water-soluble pectins occurred during ripening of wild-type but not of PG-antisense fruit.

Overall, results suggest that ripening-related pectin solubilization is highly restricted *in vivo* and largely PG-independent. The results of analysis of apoplastic soluble polymers thus support the notion of PG-independent pectin solubilization reported by other authors (Smith et al. 1990). Pectin solubilization in chill-injured fruit is more restricted than in ripening fruit, suggesting different types of pectin metabolism.

Summary

In vivo pectin solubilization was examined in ripening and chill-injured tomato fruit with down-regulated polygalacturonase (PG) activity and wild-type using a pressure-extracted apoplastic fluid. Pectin levels in the apoplastic fluid increased 3-fold during ripening and were not affected by PG levels. In contrast, PG strongly affected pectin levels in bulk pericarp fluid. There was a 14-fold increase in bulk pectin levels during ripening of PG-antisense fruit and a 36-fold increase in wild-type. Pectins in the apoplastic fluid decreased slightly during storage of fruit at 5 °C for 14 days but increased considerably upon subsequent transfer to 15 °C. Levels of monomeric galactose in the apoplastic fluid increased during ripening from 41 to 67 $\mu\text{g mL}^{-1}$. Bulk levels of galactose were 3 to 4 fold higher than apoplastic levels. Low-temperature storage caused a 50% reduction in bulk galactose levels and a 20% reduction in apoplastic levels. These results indicate that *in vivo* pectin solubilization is highly restricted and largely independent of PG. Low-temperature storage reduces *in vivo* pectin solubility, an effect that is reversed upon transfer to higher temperature following cold storage.

CHAPTER 8

POLYGALACTURONASE-MEDIATED SOLUBILIZATION AND DEPOLYMERIZATION OF PECTINS IN TOMATO FRUIT CELL WALLS IN BUFFERS MIMICKING THE pH AND MINERAL COMPOSITION OF TOMATO FRUIT APOPLAST

Introduction

Polygalacturonase (PG, EC 3.2.1.15) is the major pectin depolymerase in ripening tomato fruit. The catalytic activity of tomato PG has been the subject of extensive biochemical and molecular characterization. Ripe transgenic tomato fruit expressing negligible PG protein have lower quantities of water-soluble pectins (Carrington et al., 1993), but similar levels of chelator-soluble pectins (Smith et al., 1990) compared with untransformed fruit. Pectins from transgenic fruit have a higher degree of polymerization (Smith et al., 1990; Chapter 3).

In *in vitro* reactions, PG2 is most active at pH 4.5 and 250-300 mM NaCl (Pressey and Avants, 1973). Under these assay conditions, PG2 mediates extensive solubilization and depolymerization of pectin from isolated cell walls. PG is capable of producing pectic oligomers with a degree of polymerization lower than 10 from isolated tomato cell walls incubated in sodium acetate, pH 4.5 (Huber and Lee, 1988). Although Huber and O'Donoghue (1993) were unable to detect pectic oligomers in ethanol-

insoluble solids from ripe tomato fruit, Melotto et al. (1994) detected oligomers with a degree of polymerization ranging from 4 to 12.

Despite the information obtained through biochemical and molecular studies, the role of PG activity in ripening tomato and other fruits remains a matter of debate. Molecular silencing of PG failed to prevent softening of tomato pericarp, and a noticeable effect on firmness was noted only in the latter stages of ripening (Kramer et al., 1992; Carrington et al., 1993, Chapter 3). Chelator-soluble pectin levels are not affected by PG levels (Giovannoni et al., 1989; Smith et al., 1990). Pectin depolymerization occurs in *rin* fruit as a result of expression of a chimeric PG gene; however, further increases in PG levels during senescence do not result in additional molecular mass downshift (DellaPenna et al., 1990). Pectin solubilization in ripening tomato fruit is limited in comparison with the catalytic potential of PG present (Seymour et al., 1987).

The apoplastic conditions of ripening fruit could provide a means for regulating the activity of cell wall enzymes and help explain the discrepancies between *in vitro* catalytic potential and *in vivo* activity of PG (Huber and O'Donoghue, 1993; Chun and Huber, 1998). The apoplastic conditions of ripening fruit are not well characterized, but they are likely to be substantially different from the conditions used to assay PG *in vitro*. Ugalde et al. (1988) reported that the apoplastic pH of unripe peach was about 7 and declined to about 4.2 in ripe fruit. In apricot, apoplastic pH decreased during ripening from values higher than 6 to about 3.5 (Ugalde et al., 1988). Ruan et al. (1995) reported a pH of 6 for the apoplastic solution of immature tomato fruit.

In a previous chapter, data were presented indicating that the pH of pressure-exuded apoplastic fluid decreased from 6.7 in mature-green fruit to 4.4 in ripe fruit,

whereas K^+ levels increased from 13 to 37 mM between the same ripening stages. Ca^{2+} and Mg^{2+} remained relatively constant during ripening at about 5 and 9-13 mM, respectively (Chapter 5). These levels of divalent cations are sufficient to inhibit pectin solubilization from cell walls in vitro (Rushing and Huber, 1987) and are likely to affect the catalytic activity of pectic enzymes in vivo. PG activity is also strongly affected by ionic strength. Whereas PG activity in vitro is strongly promoted by ionic strength (NaCl) over the range of 0 to 250 mM (Pressey and Avants, 1973), the total concentration of major minerals in apoplastic fluid is comparatively low, ranging from 40 to 70 mM (Chapter 5). The objective of the present study was to examine PG-mediated pectin solubilization and depolymerization under conditions mimicking the pH and mineral composition of the apoplast of tomato pericarp at the mature-green and ripe stages of development.

Material and Methods

Plant material

Mature-green fruit were harvested from field-grown tomatoes (*Lycopersicon esculentum* Mill. cv. Solar Set) in Gainesville, FL. The fruit were surface sterilized with 2 mM NaOCl, rinsed, and air-dried. Outer pericarp sections were excised from mature-green fruit and stored at $-30\text{ }^{\circ}\text{C}$ until processed.

Cell wall preparation

Frozen peeled pericarp (50 g) was homogenized in cold 80% ethanol for 2 periods of 1 minute each using a Kinematica homogenizer (CH-6010, Luzern, Switzerland). The homogenates were stored overnight at -20°C and subsequently processed by three different methods. The three protocols were designed to yield two forms of enzymically inactive walls and enzymically active walls. Two protocols were used to isolate enzymically inactive walls to assure that differences between active and inactive walls were due to endogenous enzymes and not to the inactivation procedure. For the preparation of enzymically inactive walls, the ethanolic homogenates were filtered through Miracloth (Calbiochem Corporation, La Jolla, CA) under aspiration. The solid residue was suspended in 100 mL Tris-buffered phenol for 1 h at room temperature to inactivate enzymes (Huber, 1991). After filtration, the residue (buffered phenol inactivated walls, BP-CW) was suspended in chloroform:methanol (1:1, v/v) for 30 min. The organic solvents were removed by filtration through Miracloth and the residue was washed sequentially under aspiration with acetone (200 mL), 80% ethanol (200 mL), 40 mM HEPES, pH 7.0 (200 mL), distilled water (200 mL), 80% ethanol (100 mL), and acetone (200 mL). The residue was dried overnight at 40°C and stored in a dessicator at room temperature.

Hot-ethanol inactivated cell walls (EtOH-CW) were prepared as described for BP-CW, except that the ethanolic homogenate was refluxed in a water bath for 20 min and the BP treatment and chloroform:methanol wash were not performed.

Enzymically active cell wall material was prepared as described for BP-CW, except that the buffered phenol and chloroform:methanol treatments were omitted.

Total uronic acids and mineral content of isolated cell walls

Total uronic acids in the cell wall preparations were determined by the method of Ahmed and Labavitch (1977). For the determination of cell wall mineral content, 20 mg of cell wall material were ashed in a muffle furnace at 550 °C for 10 h. The residue after ashing was suspended in 1 mL of 6 M HCl at room temperature (about 24 °C) for 30 min, after which time the volume was brought to 10 mL with ddH₂O. Ca²⁺, Mg²⁺, K⁺, Na⁺ and P were determined by inductively coupled argon plasma spectrometry.

PG2 purification, and conditions for PG2-mediated catalysis of isolated cell walls

PG2 was purified from ripe Solar Set tomato fruit according to the protocol of Ali and Brady (1982). Maximal PG activity is achieved in sodium acetate, pH 4.5 with about 250 mM NaCl (Pressey and Avants, 1973) and variations of these conditions have been widely used for assaying PG from many fruit types. Catalysis conditions consisted of a conventional buffer for assaying PG (30 mM sodium acetate, 150 mM NaCl, pH 4.5), and solutions mimicking the pH and mineral composition of the apoplast of mature-green and ripe fruit, as determined in Chapter 5. These reaction conditions are hereafter referred to as conventional conditions (CC), mature-green conditions (MGC), and ripe conditions (RC), respectively. MGC consisted of 5 mM CaCl₂, 9 mM MgCl₂·6H₂O, 11.5 mM KCl, 6.5 mM NaOH, 1.5 mM KH₂PO₄, pH 6.5. RC consisted of 5 mM calcium acetate, 13 mM MgCl₂·6H₂O, 27.4 potassium acetate, 6.5 mM NaOH, 4.8 mM K₂HPO₄, pH 4.5. The conductivity of the solutions was measured with a YSI conductance bridge (model 31A) equipped with a model 3403 conductivity cell (Yellow Springs, Ohio) and found to be

3.1, 4.4 and 11.0 dS m⁻¹, for the MGC, RC and CC, respectively. Osmolality measured with a Wescor vapor pressure osmometer (model 5500, Logan, UT) was 125, 170 and 335 mmol kg⁻¹ for the MGC, RC and CC, respectively.

Cell walls (20 mg) were hydrated in 16 mL of the incubation solutions in an ice bath for 30 min. Afterward, the suspensions were treated with 3.8 µg of tomato PG2 (in 20 µL of 5 mM sodium acetate, 20 mM NaCl, pH 6.0) and maintained in a circulating water bath at 34 °C for 2 h. Some treatments were designed to simulate the changes in apoplastic pH and [K⁺] during ripening, hereafter designated dynamic conditions (DC). Cell walls were hydrated as described above, treated with 3.8 µg PG2 and 17 µg orange peel pectinmethylesterase (PME, EC 3.1.1.11, Sigma, solubilized in distilled water, 120 µL added to reaction mixture), and incubated in MGC at 34 °C for 2 h. After this incubation period the pH was adjusted to 4.5 with dilute HCl, and K⁺ increased with the addition of potassium acetate to simulate the conditions of RC. The cell walls were incubated for an additional 2 h. The pH of the suspensions was measured after initial cell wall hydration and at the end of the incubation period.

Post catalysis extraction and chromatography of pectins

After the catalysis periods, the cell wall suspensions were chilled in an ice-bath and filtered through Whatman GF/C filter paper under aspiration into a tube containing 400 µL of 500 mM Tris, pH 7.5 to arrest PG activity. The filtrate was assayed for uronic acids (Blumenkrantz and Asboe-Hansen, 1973) and reducing groups from hexuronic acids (Milner and Avigad, 1967). Neutral sugars in the filtrates were determined as described in Chapter 7.

After catalysis and filtration, the cell walls were suspended in 15 mL 95% ethanol, refluxed for 15 min to inactivate residual enzymes, and then stored at -20 °C until analyzed. The cell walls were recovered by centrifugation at 2000g for 20 min, the supernatant discarded, and the pellet dried at 34 °C. Chelator-soluble pectins were extracted by suspending 10-15 mg of post-incubation, heat-inactivated cell walls in 7 mL 50 mM sodium acetate, 50 mM CDTA, pH 6.5 (Jarvis et al., 1981) and incubated for 6 h at 24 °C with shaking. The suspension was then filtered through a Whatman GF/C filter and total uronic acids determined in the filtrate. CDTA-soluble pectins were separated by size on a Sepharose CL-4B column (29 cm long, 1.5 cm diameter) operated with a mobile phase of 200 mM ammonia acetate, pH 5.0 (Mort et al., 1991).

Results

Pectin and mineral content of cell wall preparations

The yield of cell wall material was 23.2, 26.5 and 27.5% of pericarp dry weight for BP-CW, EtOH-CW, and active walls, respectively. Total uronic acids averaged 289, 241 and 288 $\mu\text{g mg}^{-1}$ for BP-CW, EtOH-CW, and active cell walls, respectively.

Na^+ and Ca^{2+} were the most abundant cations in cell walls prepared by each isolation protocol (Table 8-1). EtOH-CW retained higher levels of Ca^{2+} , Mg^{2+} and P but less Na^+ than the BP-CW and active walls (Table 8-1).

Table 8-1. Mineral content of cell wall preparations from mature-green tomato pericarp. Values are means $\pm$ SE of 3 observations.

CW	Ca	Mg	K	P	Na
	$\mu\text{g (mg cell wall)}^{-1}$				
Active ^z	4.58 $\pm$ 0.03	1.42 $\pm$ 0.01	0.26 $\pm$ 0.03	0.38 $\pm$ 0.01	9.47 $\pm$ 1.02
BP-CW ^y	4.83 $\pm$ 0.05	1.21 $\pm$ 0.01	0.22 $\pm$ 0.02	0.39 $\pm$ 0.00	12.81 $\pm$ 1.97
EtOH-CW ^x	5.73 $\pm$ 0.02	1.77 $\pm$ 0.02	0.26 $\pm$ 0.03	0.67 $\pm$ 0.01	5.80 $\pm$ 0.05

z, Enzymically active cell walls

y, Phenol inactivated cell walls

x, Hot ethanol inactivated cell walls

pH changes during catalysis

Due to the weak buffering capacity of the MGC solution, there was a slight decrease in pH of the cell wall suspension from the original pH of 6.5 to 6.3 during the hydration period. The pH of the CC and RC solutions did not change during the hydration period. During catalysis, the pH of the cell wall suspensions remained within 0.1 units of the post-hydration pH in all incubation media. Incubation of BP-CW in the MGC with PG and PME for 2 h was accompanied by the release of H^+ at a rate of 14 nmol min^{-1} , causing a drop in pH from 6.3 to 5.7. Active cell walls with PG and PME released H^+ at a rate of 3 nmol min^{-1} , decreasing the pH from 6.3 to 6.1. At the end of the 2-hour incubation in the MGC, the suspension was titrated to the characteristics of ripe apoplast (adjusting the pH to 4.5 and increasing K^+ to 37 mM) and incubated for an additional 2 h. No pH changes were observed during this period.

Effect of catalysis medium on PG-mediated pectin solubilization

Solubilization of uronic acids and the production of reducing groups from cell walls of mature-green tomato fruit were strongly influenced by the composition of the catalysis medium (Figures 8-1 through 8-4). In the absence of PG, enzymically inactive cell walls incubated in 30 mM NaOAc, 150 mM NaCl, pH 4.5 (CC) released 4.9 (BP-CW) or 12 (EtOH-CW) $\mu\text{g mg}^{-1}$ of uronic acids, values 8 and 20-fold higher for BP-CW and EtOH-CW, respectively, than enzymically active cell walls (Figure 8-1A). PG-treated cell walls released 86.4, 72.3, and 45.7 $\mu\text{g mg}^{-1}$ of uronic acids, for BP-CW, EtOH-CW and active walls, respectively (Figure 8-1A). PG induced a 6-fold increase in uronate solubilization from EtOH-CW, an 18-fold increase from BP-CW, and a 77-fold increase from active cell walls. In the absence of PG, the release of reducing groups was higher in active (1.7 $\mu\text{g mg}^{-1}$) than in inactive cell walls (0.1 and 0.5 $\mu\text{g mg}^{-1}$ for BP-CW and EtOH-CW, respectively), in contrast with uronic acid solubilization, which was higher in inactive walls. PG strongly increased the amount of reducing groups, indicating pectin depolymerization (Figure 8-1B).

When compared with cell walls incubated under CC, pectin solubilization from all cell wall isolates was very restricted in simulated apoplastic solutions. In cell walls incubated under MGC, pectin solubilization ranged from 0.3 to 0.8 $\mu\text{g mg}^{-1}$ and was unaffected by PG (Figure 8-2A). The release of reducing groups from cell walls in MGC was low and PG-independent. Despite the very low yields, active walls released 2.7 $\mu\text{g mg}^{-1}$ of reducing groups, twice the level as inactive cell walls (Figure 8-2B).

In the absence of added PG, both active and inactive cell walls incubated in a solution with the mineral composition of ripe fruit apoplastic fluid (RC) released lower

quantities of uronic acid compared with incubation in MGC. Under RC, addition of PG to the reaction mixture resulted in the release of 0.8, 7.4, and 18.3 $\mu\text{g mg}^{-1}$ of uronic acid for active, EtOH-CW, and BP-CW, respectively. PG greatly increased pectin solubilization but, in contrast with CC, the active walls showed only a 6-fold increase compared with 25 and 36-fold increase from EtOH-CW and BP-CW, respectively (Figure 8-3A). PG-mediated solubilization in RC was very restricted compared with incubation under CC: only 2% of the release in CC for active walls and 9 and 25 % for BP- and EtOH-inactivated walls, respectively. PG did not affect the release of hexuronic acid reducing groups from active or EtOH-CW, although some increase in release was observed in BP-CW (Figure 8-3B).

The lower PG-mediated solubilization of pectin from active walls compared with inactive walls might be caused by increased negative charges generated by PME action during the preparation and incubation of active walls. To test this idea, reactions were carried out in MGC for 2 h, after which the solution pH and $[\text{K}^+]$ were adjusted to that of the RC. At the beginning of incubation, reaction suspensions were provided with tomato PG2 (3.8 μg), with or without orange PME (17 μg). As shown in figure 8-4A, the presence of PME in the reaction mixtures suppressed the capacity of PG to solubilize uronic acids from both phenol and hot-ethanol inactivated cell wall preparations by 86 and 88%, respectively. Active walls showed negligible pectin release in response to enzyme treatment under these reaction conditions.

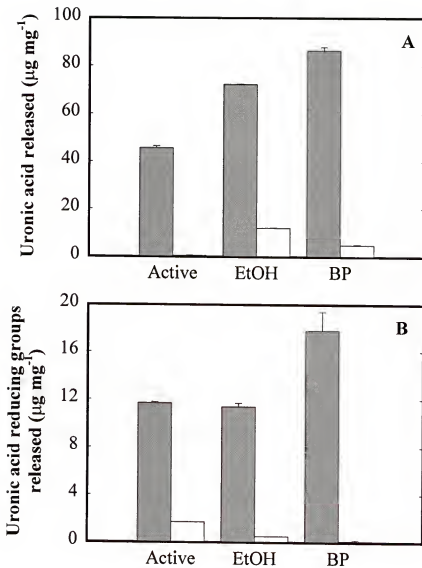


Figure 8-1. Uronic acid and reducing groups released from cell walls from mature-green tomato fruit incubated with (gray bars) or without (white bars) PG under conventional conditions (CC, 30 mM NaOAc, 150 mM NaCl, pH 4.5) for 2 h at 34 °C. A, Uronic acid released; B, uronic acid reducing groups released. Values are means $\pm$ SE of 2 observations.

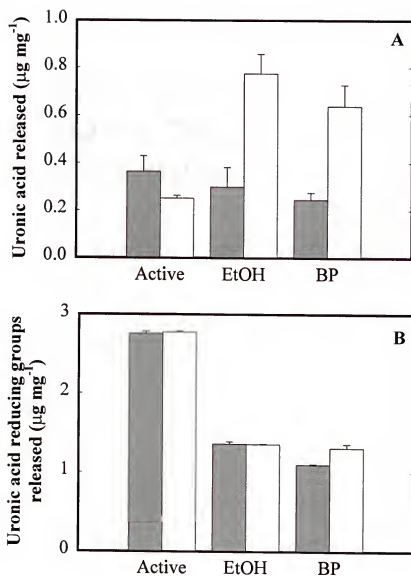


Figure 8-2. Uronic acid and reducing groups released from cell walls from mature-green tomato fruit incubated with (gray bars) or without (white bars) PG under mature-green conditions (MGC) for 2 h at 34 °C. A, Uronic acid released; B, uronic acid reducing groups released. Values are means $\pm$ SE of 2 observations.

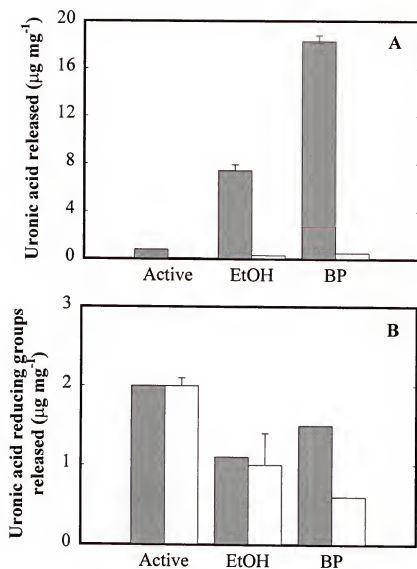


Figure 8-3. Uronic acid and reducing groups released from cell walls from mature-green tomato fruit incubated with (gray bars) or without (white bars) PG under ripe apoplastic conditions (RC) for 2 h at 34 °C. A, Uronic acid released; B, uronic acid reducing groups released. Values are means $\pm$ SE of 2 observations.

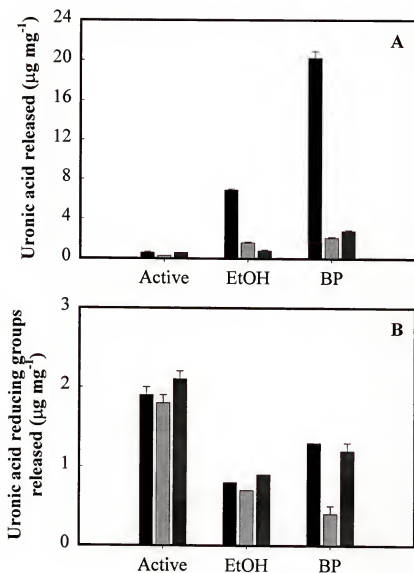


Figure 8-4. Uronic acid and reducing groups released from cell walls from mature-green tomato fruit incubated with PG (black bars) or with PG and PME (dark gray bars) or without added enzymes (light gray bars) under "dynamic apoplastic conditions" (DC). A, Uronic acid released; B, uronic acid reducing groups released. Values are means $\pm$ SE of 2 observations.

The presence of Ca^{2+} at 5 mM in the MGC and RC may account for the low quantities of pectins released from cell walls. BP-CW incubated in 5 mM calcium (as calcium acetate), pH 4.5 in the presence of PG released $13 \mu\text{g}.\text{mg}^{-1}$ of uronic acid compared with the $18 \mu\text{g}.\text{mg}^{-1}$ released under RC.

Neutral sugar composition of the polymers released from cell walls

Table 8-2 presents the concentration of neutral sugars released during incubation of active and BP-inactivated cell walls under the three reaction conditions. In BP-CW incubated under CC, MGC, and RC, PG increased solubilization of neutral sugars compared with nontreated controls, but the effect of PG was less evident in active cell walls. The highest release of neutral sugars was generally noted for cell walls that were incubated under conditions that simulate the changes in apoplastic pH and $[\text{K}^+]$ during ripening, possibly due to the extended incubation period. Galactose was the most abundant neutral sugar released, followed by glucose and mannose (Table 8-2).

Gel filtration profiles of pectins extracted post incubation by CDTA

In view of the low levels of soluble pectins released from cell walls incubated in simulated apoplastic solutions, cell walls following catalysis were filtered, treated with hot ethanol (to inactivate enzymes used for catalysis or associated with active walls) and subsequently extracted with 50 mM CDTA in 50 mM sodium acetate, pH 6.5. The objective was to determine if added enzymes were capable of depolymerizing pectins independently of pectin solubilization.

Table 8-2. Neutral sugars released from cell walls (nmol mg⁻¹) during incubation in various solutions.

Solution	CW	Enzyme	Rha	Ara	Xyl	Man	Glc	Gal	Total
CC	Active	No	0.8	4.1	5.2	9.5	9.1	11.2	39.9
CC	Active	PG	2.8	9.4	4.0	5.2	4.3	15.8	41.5
MGC	Active	No	0.3	2.1	5.5	6.5	7.7	8.1	30.2
MGC	Active	PG	0.5	0.0	5.3	5.9	6.6	7.0	25.4
RC	Active	No	0.7	3.9	6.9	9.3	8.9	9.4	39.2
RC	Active	PG	1.0	5.0	10.0	9.9	9.9	12.6	48.5
DC	Active	No	2.1	5.2	5.6	9.1	7.8	10.7	40.5
DC	Active	PG	1.6	5.9	7.6	10.7	10.8	13.3	49.9
DC	Active	PG+PME	2.0	10.7	9.8	14.3	15.0	17.0	68.8
CC	BP	No	1.3	4.0	5.5	8.1	7.1	10.1	36.1
CC	BP	PG	2.8	10.6	4.3	4.9	3.6	18.8	45.0
MGC	BP	No	0.9	3.4	8.4	11.4	13.3	10.8	48.3
MGC	BP	PG	2.0	2.8	8.5	10.7	11.6	9.8	45.4
RC	BP	No	0.4	2.2	3.2	4.0	3.3	4.4	17.3
RC	BP	PG	0.0	4.5	6.3	7.5	7.2	9.3	34.8
DC	BP	No	0.9	1.4	2.1	2.1	2.1	2.7	11.4
DC	BP	PG	1.2	4.7	12.1	10.9	11.1	11.2	51.2
DC	BP	PG+PME	7.3	11.9	34.1	14.0	15.8	13.7	96.9

Table 8-3. Uronic acids ($\mu\text{g mg}^{-1}$) released during catalysis (Cat), extracted in CDTA (50 mM CDTA, 50 mM sodium acetate, pH 6.5) from cell wall residues after catalysis, and total uronic acids recovered from cell walls. Cell walls were incubated under conventional conditions (CC), mature-green conditions (MGC), and ripe conditions (RC). Values are means $\pm$ SE of 2 observations

CW	Enzyme	CC			MGC			RC		
		Cat.	CDTA	Total	Cat.	CDTA	Total	Cat.	CDTA	Total
Active ^z	PG	45.7 $\pm$ 0.8	20.5 $\pm$ 5.6	66.3 $\pm$ 6.4	0.4 $\pm$ 0.1	47.9 $\pm$ 6.0	48.3 $\pm$ 6.0	0.8 $\pm$ 0.0	44.1 $\pm$ 3.1	44.9 $\pm$ 3.0
	Control	0.6 $\pm$ 0.1	6.2 $\pm$ 0.3	6.8 $\pm$ 0.4	0.3 $\pm$ 0.0	48.7 $\pm$ 7.3	49.0 $\pm$ 7.3	0.1 $\pm$ 0.0	30.1 $\pm$ 1.1	30.2 $\pm$ 1.1
BP-CW ^y	PG	86.4 $\pm$ 1.4	41.4 $\pm$ 0.3	127.7 $\pm$ 1.8	0.2 $\pm$ 0.0	67.1 $\pm$ 9.9	67.4 $\pm$ 2.0	18.3 $\pm$ 0.5	92.7 $\pm$ 5.3	110.9 $\pm$ 5.8
	Control	4.9 $\pm$ 0.3	29.4 $\pm$ 0.1	34.3 $\pm$ 0.2	0.6 $\pm$ 0.1	53.9 $\pm$ 2.1	54.5 $\pm$ 6.0	0.5 $\pm$ 0.0	94.1 $\pm$ 0.6	94.7 $\pm$ 0.6
EtOH-CW ^x	PG	72.3 $\pm$ 0.0	48.6 $\pm$ 3.4	121.0 $\pm$ 3.2	0.3 $\pm$ 0.1	77.7 $\pm$ 8.0	78.0 $\pm$ 8.0	7.4 $\pm$ 0.5	89.3 $\pm$ 8.4	96.7 $\pm$ 7.8
	Control	12.0 $\pm$ 0.1	53.9 $\pm$ 2.1	65.9 $\pm$ 5.6	0.8 $\pm$ 0.1	57.4 $\pm$ 0.4	58.2 $\pm$ 0.3	0.3 $\pm$ 0.0	88.0 $\pm$ 3.5	88.3 $\pm$ 3.5

z, Enzymically active cell walls

y, Phenol inactivated cell walls

x, Hot ethanol inactivated cell walls

Table 8-4. Uronic acids ($\mu\text{g mg}^{-1}$) released during catalysis (Cat), extracted in CDTA (50 mM CDTA, 50 mM sodium acetate, pH 6.5) from cell wall residues after catalysis, and total uronic acids recovered from cell walls incubated in MGC for 2 h, after which the solution pH and $[\text{K}^+]$ were adjusted to that of the RC. Values are means $\pm$ SE of 2 observations

CW	Enzyme	Cat	CDTA	Total
Active ^z	PG	0.6 $\pm$ 0.1	122.5 $\pm$ 6.6	123.1 $\pm$ 6.6
	No	0.3 $\pm$ 0.0	105.6 $\pm$ 8.0	105.9 $\pm$ 7.9
	PG+PME	0.6 $\pm$ 0.0	161.8 $\pm$ 28.6	162.4 $\pm$ 28.6
BP-CW ^y	PG	20.2 $\pm$ 0.7	120.5 $\pm$ 7.8	140.6 $\pm$ 8.5
	No	2.1 $\pm$ 0.1	125.2 $\pm$ 3.4	127.3 $\pm$ 3.5
	PG+PME	2.8 $\pm$ 0.1	173.8 $\pm$ 2.1	176.6 $\pm$ 2.2
EtOH-CW ^x	PG	6.9 $\pm$ 0.1	129.2 $\pm$ 4.2	136.1 $\pm$ 4.2
	No	1.6 $\pm$ 0.1	111.6 $\pm$ 3.8	113.3 $\pm$ 3.9
	PG+PME	0.8 $\pm$ 0.1	153.4 $\pm$ 26.1	154.2 $\pm$ 26.1

z, Enzymically active cell walls

y, Phenol inactivated cell walls

x, Hot ethanol inactivated cell walls

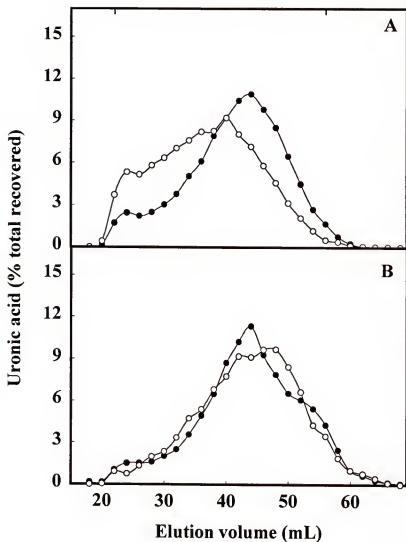


Figure 8-5. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after incubation with (●) or without (○) PG under conventional conditions (CC; 30 mM sodium acetate, 150 mM NaCl, pH 4.5). A, BP-inactivated walls; B, enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

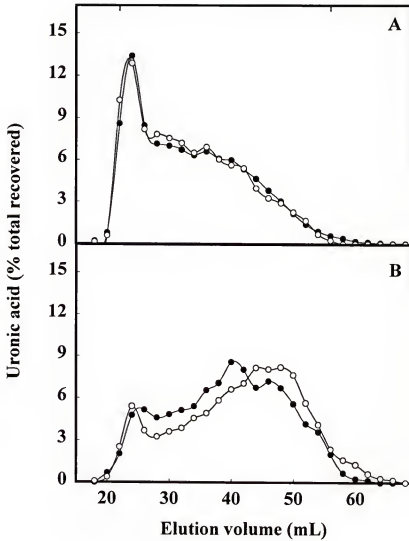


Figure 8-6. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after incubation with (●) or without (○) PG under mature-green apoplastic conditions (MGC). A, BP-inactivated walls; B, enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

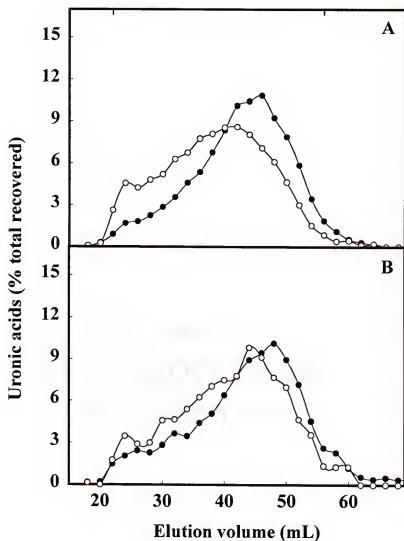


Figure 8-7. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after incubation with (●) or without (○) PG under ripe apoplastic conditions (RC). A, BP-inactivated walls; B, enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

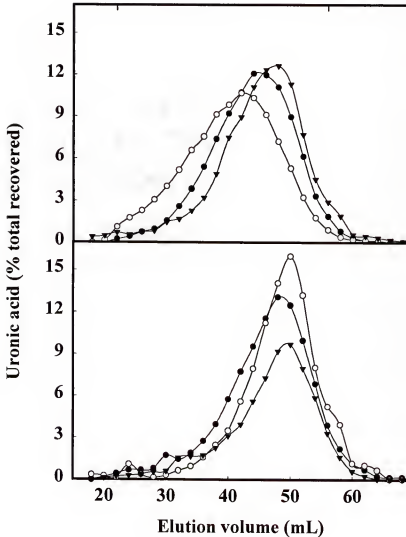


Figure 8-8. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after incubation with PG(●), PG and PME (▼) or without added enzymes (○) under "dynamic apoplastic conditions" (DC). A, BP-inactivated walls; B, enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

Table 8-3 presents a summary of the recovery of cell wall uronic acids during catalysis reactions and from subsequent extraction in CDTA after incubation under the three incubation conditions. Under all incubation conditions, active cell walls yielded less CDTA-soluble pectins than either BP-CW or EtOH-CW, although the difference was larger under CC and RC than in MGC.

Gel filtration profiles of CDTA-soluble pectins recovered from cell walls derived from the active and BP-CW first incubated under CC are shown in figure 8-5. CDTA-soluble pectins recovered from BP-CW treated with PG were of lower molecular mass than polymers recovered from the same walls without PG (Figure 8-5A). CDTA-soluble pectin recovered from active walls incubated in CC with or without PG were similar in molecular mass distribution (Figure 8-5B) and comparable to BP-CW treated with PG.

The molecular size distribution of pectins released from BP-CW incubated in MGC was similar in walls treated with or without PG, with a major peak at the void volume (Figure 8-6A). Active walls incubated under MGC, with or without PG, released pectins with a lower average molecular mass and higher polydispersity than the pectins released from BP-CW (Figure 8-6B).

Gel filtration profiles of CDTA-soluble pectins recovered from phenol-inactivated cell walls following treatment with purified PG under RC are shown in Figure 8-7A. These polymers were of lower molecular mass than those recovered from BP-CW incubated in the absence of PG. CDTA-soluble polymers recovered from active walls previously incubated in RC with or without PG were similar in molecular mass distribution (Figure 8-7B).

Total pectin recovery was highest (162 and 177 $\mu\text{g mg}^{-1}$ in active and BP-CW, respectively) in walls treated with both PG and PME, with nearly all pectins being in the CDTA-soluble fraction (Table 8-4). CDTA-soluble pectins from BP-CW incubated under DC with PG had lower molecular mass than those incubated in the absence of PG. The presence of PG and PME in the incubation solution resulted in a further decrease of molecular mass of CDTA-soluble pectins than PG alone (Figure 8-8A). CDTA-soluble polymers recovered from active walls incubated under DC with PG and PME were similar in molecular mass distribution to polymers recovered from active cell walls incubated with PG alone or without added enzymes (Figure 8-8B).

Discussion

The results presented show that pectin solubilization from cell walls is strongly restricted in solutions with the pH and mineral composition of the apoplast of ripening tomato fruit. Conventional buffers (30 mM sodium acetate, 150 mM NaCl, pH 4.5) have higher ionic strength than the apoplastic solution of ripening tomato fruit. The osmolality of the apoplastic solution of tomato pericarp is in the range 65-95 mmol kg^{-1} for mature-green fruit and 130 to 140 mmol kg^{-1} for ripe fruit (Chapter 5), much lower than the 335 mmol kg^{-1} for 30 mM sodium acetate, 150 mM NaCl. Data presented in Chapter 7 showed that *in vivo* pectin solubilization in ripening tomato pericarp is highly restricted. The present data supports a role for the mineral composition of the apoplast in regulating pectin solubilization in ripening tomato. Solubilization was lower in the simulated apoplastic solutions than in 30 mM sodium acetate with 150 mM NaCl for both active and inactive walls, with or without PG. The restricted pectin solubilization can be

partially explained by the levels of divalent cations present in the simulated apoplastic solutions. Ca^{2+} at the levels used in this study (5 mM) strongly limits the autolysis of ripe tomato cell wall (Rushing and Huber, 1987), and may account for the restricted pectin solubilization in simulated apoplastic solutions.

PG activity can be modulated by the ionic strength and mineral composition of the incubation solutions. The activity of PG is greatly enhanced by NaCl at 250 mM (Pressey and Avants, 1973). In 250 mM NaCl, PG2 is active over the pH range from 3.5 to 5.5, but activity in water is substantially lowered and restricted to the pH range 5 to 5.5 (Pressey and Avants, 1973). When K^+ replaces Na^+ as the major cation, the pH optimum for PG2 activity is 1.5 and 0.5 units higher for polygalacturonic acid and cell walls, respectively (Chun and Huber, 1998).

Pectin solubilization and depolymerization are independent processes. Ripening-related pectin solubilization may occur in the absence of PG, as in the case of strawberry (Huber, 1984), persimmon (Cutillas-Iturralde et al., 1993), and tomato locule tissue (Cheng and Huber, 1996). In tomato pericarp, *in vivo* pectin solubilization is largely PG-independent (Chapter 4 and 7). PG stimulated the release of reducing groups from cell walls incubated in 30 mM sodium acetate with 150 mM NaCl, but not under simulated apoplastic conditions (except for BP-CW in RC). However, analysis of the molecular mass distribution of ionically bound pectins revealed that PG depolymerized CDTA-soluble pectins in inactive walls incubated in CC and RC. The results show that PG is capable of depolymerizing ionically bound pectins without solubilization.

Pectin solubilization from active walls was consistently lower than that from inactive walls. This may be partially attributed to the presence of the PG β -subunit in

active walls. This protein is present in the cell walls of mature-green tomato fruit and restricts PG-mediated solubilization of pectins (Watson et al., 1994). Under the conditions used in this study, however, the presence of PME in active walls seems to account for most of the difference in pectin release from active walls vs. inactive walls. PME activity enhances Ca^{2+} binding to the cell walls (Tiemann and Handa, 1994) and can convert trichloroacetic acid-soluble pectins into an insoluble form (Pressey, 1984). Since PME activity is highest at the mature-green stage (Gaffe et al., 1994), the possibility was tested that the enzyme may have been active during the preparation and incubation of active walls, explaining the restricted solubilization of pectins from active walls. The 3 PME isozymes present in ripening tomato have optimal activity at pH values higher than 6.5, with only one isoform retaining substantial activity at pH values lower than 5 (Giovane et al., 1994; Warrilow and Jones, 1995). In contrast, PG2 has optimal activity at pH 4.5-5 and is inactive at pH 6 and above (Pressey and Avants, 1973). Under low ionic strength, the pH optima for both PG (Pressey and Avants, 1973) and PME (Pressey and Avants, 1972; Pressey and Woods, 1992) are shifted to higher values and the enzymes are active over a narrower pH range. The ripening-related changes in apoplastic pH and K^+ levels were simulated by incubating cell walls from mature-green fruit in MGC for 2 h, after which the solution was titrated to RC and incubated for an additional 2 h. Under these conditions, BP-CW treated with PG released similar quantities of uronic acids than when the same walls were incubated under RC, indicating that negligible levels of uronic acid were released during the initial 2 h incubation period (under MGC). Pectins recovered from BP-CW under DC had molecular mass distribution similar to that of pectins recovered under RC. The presence of PME inhibited PG-mediated

solubilization (Figure 8-4A) and enhanced depolymerization of the ionically bound pectins (Figure 8-8A). In contrast, Pressey and Avants (1982) showed that PME enhanced PG-mediated solubilization of pectins from mature-green tomato cell walls in 100 mM glycine-acetate, pH 5. At pH 3.5, however, low levels of PME enhanced pectin solubilization whereas higher PME levels inhibited pectin solubilization. Since PME was three times more active at pH 5 than 3.5, Pressey and Avants (1982) suggested that the degree of esterification optimal for PG activity is lower at pH 5 than at pH 3.5. The levels of Ca^{2+} found in cell wall preparations (Table 8-1) account, on a molar basis, for 8% of the total uronic acid in active and BP-CW and 11.5% of total uronic acid in EtOH-CW. Incubating acetone insoluble solids from mature-green tomato in 40 mM EDTA, 50 mM sodium acetate, pH 4.5, conditions that would remove cell wall Ca^{2+} , Seymour et al. (1987) reported that PME enhanced PG-mediated solubilization and depolymerization of pectin from mature-green tomato.

CDTA-soluble pectins from BP-CW incubated under MGC showed a large peak of high molecular mass polymers eluting in the void volume (Figure 8-6A). When the same walls were incubated under RC, the resulting CDTA-soluble pectins showed a molecular mass downshift, an effect accentuated by the presence of PG (Figure 8-7A). These profiles reflect molecular mass downshifts similar to the ones observed during ripening of tomato fruit (Figure 3-4 in Chapter 3). However, pectins extracted from inactive walls incubated under RC in the absence of PG were also of lower mass than the same walls incubated under MGC. In contrast, CDTA-soluble pectins from ripe PG-antisense fruit showed limited reduction in molecular mass compared with pectins from mature-green fruit (Figure 3-4 in Chapter 3). Active walls, on the other hand, yielded

similar quantities of CDTA-soluble pectins that showed similar profiles under MGC and RC, independently of the presence of PG. The incubation conditions affected the population of polymers susceptible to extraction by CDTA (Table 8-3 and 8-4). Moreover, the yields and profiles of pectins extracted from active walls were different than those from inactive walls incubated under the same conditions suggesting that enzymes other than PG might play a role in pectin solubilization and dissociation.

In ripening tomato, *in vivo* pectin solubilization is restricted in relation to the potential *in vitro* activity of PG present (Seymour et al., 1987). Contradictions between *in vitro* catalytic potential and alterations observed *in vivo* were also reported for β -galactosidase. In avocado (De Veau et al., 1993), kiwifruit (Ross et al., 1993) and apple fruit (Ross et al., 1994), *in vivo* loss of galactose was much higher than the *in vitro* potential of β -galactosidases. These disparities are probably applicable to other cell wall enzymes assayed under conditions that favor optimal catalysis. The results presented herein show that PG-mediated pectin solubilization and depolymerization is highly restricted under the conditions prevailing in the apoplast of ripening tomato fruit, and support the concept that the apoplastic conditions can regulate cell wall disassembly in ripening fruit.

Summary

Pectin solubilization in apoplast of ripening tomato fruit is independent of polygalacturonase (PG) and highly restricted compared to solubilization from isolated cell walls, possibly owing to the pH and mineral composition of the apoplast. The effects of PG on pectin solubilization and depolymerization were examined in mature-green

tomato fruit cell walls incubated in a conventional buffer for assaying PG activity (30 mM sodium acetate, 150 mM NaCl, pH 4.5) and in solutions mimicking the pH and mineral composition of the fruit apoplast at the mature-green and ripe stages. Under simulated apoplastic conditions pectin solubilization from cell walls is much lower than in 30 mM sodium acetate with 150 mM NaCl. PG-treated walls released more pectins than controls with no enzyme when incubated in sodium acetate buffer or simulated ripe apoplastic solution, but not in simulated mature-green solution. Release of reducing groups from cell walls was also very limited in simulated apoplastic conditions compared to sodium acetate with 150 mM NaCl. PG increased the release of reducing groups from cell wall incubated in sodium acetate with 150 mM NaCl; under simulated apoplastic conditions, however, the release of reducing groups was similar in cell wall treated with or without PG. Under most incubation conditions, pectin solubilization from active cell walls was lower than that from inactive walls due to the activity of pectinmethylesterase (PME). Gel filtration profiles of pectins solubilized in CDTA following incubation revealed that PG is capable of hydrolyzing ionically bound polymers.

CHAPTER 9

SUMMARY AND CONCLUSIONS

The primary objectives of the research reported in this study were to characterize the pH and mineral composition of the apoplast of ripening tomato fruit and to assess whether the composition of the apoplast could regulate cell wall metabolism and fruit softening. The dramatic changes that occur in pectic polymers during tomato fruit ripening, and the evidence that *in vivo* pectin metabolism differs from that reproduced in cell-free systems lead to additional characterization of the pectins in ripening tomato using a variety of approaches. Low-temperature storage, resulting in chilling injury, was used as a means to alter plasma membrane permeability and induce changes in the apoplastic environment. The pH and mineral composition of the apoplast and pectin metabolism were characterized in chill-stressed fruit. Transgenic tomato fruit with reduced polygalacturonase (PG) expression and wild-type fruit were used as a tool for studying the effects of PG on pectin metabolism. Finally, PG catalysis of isolated cell walls was studied in solutions mimicking the pH and mineral composition of the fruit apoplast and contrasted with catalysis in buffers typically used for assaying PG activity.

Apoplastic pH and Mineral Composition in Ripening Tomato Fruit

The role of pH on cell wall metabolism has long been recognized in vegetative organs in relation to extension growth and incorporated in the acid-growth theory.

However, and despite several proposals that apoplastic pH might affect fruit softening metabolism, the apoplastic conditions of ripening fruit remained largely uncharacterized.

Analysis of a pressure-extracted fluid of putative apoplastic origin revealed that the apoplastic solution of mature-green pericarp is dilute with an osmolality of 66 mOsmol kg⁻¹, pH of 6.7, and contains about 5 mM Ca²⁺, 9 mM Mg²⁺, 13 mM K⁺, 6.5 mM Na⁺, and 4 mM Cl⁻. During ripening there was an increase in apoplastic osmolality to 130 mOsmol kg⁻¹, acidification to pH 4.4, and a 3-fold increase in K⁺ levels to 37 mM, while the levels of other minerals showed minimal change.

Cell wall enzymes are regulated during ripening at the transcriptional level. In addition, the decrease in apoplastic pH and increase in ionic strength observed during fruit ripening may provide a strong means for biochemical regulation of cell wall enzymes. The patterns of accumulation of transcripts for cell wall enzymes are roughly concurrent with the occurrence of optimal pH for their activity in the apoplast. The acidification of apoplastic pH from 7 to 4.5 during ripening favors the sequential action of enzymes that disassemble the cellulose-hemicellulose and pectic networks.

Polygalacturonase Catalysis in Solutions Mimicking the Composition of the Apoplastic Fluid of Ripening Tomato

PG-mediated uronic acid solubilization from isolated cell walls was determined in 30 mM sodium acetate, 150 mM NaCl, pH 4.5 and in solutions mimicking the pH and mineral composition of the apoplast of mature-green and ripe tomato fruit. PG activity is very limited in "apoplastic" solutions compared with 30 mM sodium acetate, 150 mM NaCl. Uronic acid solubilization is virtually absent in cell walls incubated under the apoplastic conditions of mature-green fruit. PG was able to solubilize uronic acids from

cell walls under ripe apoplastic conditions, but solubilization was very restricted compared with 30 mM sodium acetate, 150 mM NaCl. Enzymically active cell walls incubated under all conditions released less pectins than inactive walls due to the activity of pectinmethylesterase. Gel filtration profiles of CDTA-soluble pectins extracted after catalysis revealed that PG is capable of hydrolyzing pectins without solubilization.

Examination of the autolytic activity of cell walls isolated from PG-antisense fruit at several ripening stages showed that, under apoplastic conditions, autolysis required PG. However, it is possible that the composition of the apoplastic fluid affects pectin solubilization by nonenzymic mechanisms.

Pericarp Softening and Pectin Disassembly in Ripening Tomato Fruit

The pericarp of PG-antisense and wild-type fruit soften at a similar rate during the initial ripening stages but, at the ripe stage, fruit with negligible levels of PG are firmer than wild-type. Softening is accompanied by increased pectin solubilization. Water-soluble pectins recovered from ethanol-insoluble solids (EIS) showed a PG-dependent increase during ripening, whereas CDTA-soluble pectins increased independently of PG levels. Pectin efflux from pericarp disks incubated in an isotonic solution (300 mM of mannitol) increased during ripening at a similar rate in PG-antisense and wild-type fruit, suggesting that the effect of PG on water-soluble pectin recovered from EIS may be an artifact of cell wall isolation. Extraction and analysis of apoplastic fluid from ripening PG-antisense and wild-type fruit revealed that *in vivo* pectin solubilization is independent of PG levels.

Water- and CDTA-soluble pectins recovered from EIS isolated from ripening tomato fruit showed that extensive reduction in molecular mass of pectic polymers occurred during ripening of wild-type fruit, whereas PG-antisense fruit exhibited very limited depolymerization. Similar differences in molecular mass downshift during ripening between the PG-antisense and wild-type fruit were observed in the gel filtration profiles of pectins passively effluxed from pericarp disks, suggesting that pectin depolymerization *in vivo* is mediated by PG.

Pericarp Softening and Pectin Disassembly in Tomato Fruit Following Low-temperature Storage

Pericarp firmness was maintained in mature-green tomato fruit during storage at 5 °C, but rapid softening occurred upon transfer of previously chilled fruit to higher temperatures (15-18 °C). In contrast with ripening-related softening, the decrease in firmness of chill-stressed fruit occurred without increases in water- or CDTA-soluble pectins (from EIS), which remained at levels comparable to those of mature-green fruit prior to low-temperature storage. Gel filtration profiles of CDTA-soluble pectins from EIS revealed no depolymerization during storage at 5 °C or during subsequent storage at 18 °C for 5 days following low-temperature storage. Similarly, pectin efflux from pericarp disks excised from chill-injured fruit and incubated in 300 mM mannitol remained at the levels found in fruit prior to low-temperature storage and showed no signs of depolymerization, indicating that *in vivo* pectin disassembly is highly restricted in chill-injured fruit compared with ripening fruit. However, disruption of the pericarp of chill-injured fruit by freezing and thawing resulted in extensive pectin depolymerization, indicating that the tissue contains the necessary depolymerases but their activity requires

loss of compartmentation. Pectin levels in the pressure-extracted apoplastic fluid decreased during storage at 5 °C (14 days) and increased after subsequent transfer of the fruit to 15 °C for 6 days to levels higher than those found in freshly harvested fruit, but lower than those found in ripe fruit. Apoplastic levels of free galactose decreased slightly during low-temperature storage.

The design of experiments for studying the effects of low temperatures in the metabolism of chilling sensitive fruits poses a methodological problem concerning control treatments. Fruit stored at temperatures below the threshold for injury are inhibited in ripening, whereas control fruit stored at temperatures above the injury threshold continue ripening at a temperature-dependent rate. Comparisons between these two situations lead to confoundment between the effects of temperature on chilling-injury and developmental stage. Alternatively, comparing fruit stored at low-temperature for a period of time with freshly harvested fruit prior to storage does not account for the different chronological ages. The inhibitor of ethylene action 1-methylcyclopropene was used to arrest ripening and allow fruit stored above the threshold for chilling injury to age without ripening thus providing more appropriate controls for studying the effects of low-temperature on tomato fruit physiology.

Apoplastic pH and Mineral Composition of Tomato Fruit Following Low-temperature Storage

Chilling injury causes softening of tomato pericarp, but cell wall disassembly in chill-injured fruit differs from ripening-related metabolism. Low-temperature storage induced changes in the putative apoplastic fluid from mature-green tomato fruit different from those found in ripening fruit. The apoplastic solution of mature-green fruit is more

dilute than the bulk solution. The osmolality of the apoplastic solution of mature-green fruit increased 25% during 14 days at 5 °C and an additional 18% increase was observed after 6 days at 15 °C following low-temperature storage. Even in chill-injured fruit, the osmolality of the apoplastic fluid was only about 50% of the value found in the bulk fluid. Low-temperature storage resulted in a decrease in the pH of the apoplastic fluid from 5.7 to 4.5 and further acidification occurred upon transfer of the fruit to 15 °C to reach 4.3 after 6 days. The levels of Ca^{2+} and Mg^{2+} in the apoplastic fluid remained constant at 5.9 and 18.1 mM, respectively, during low-temperature storage but declined about 45% following transfer to 15 °C and storage at this temperature for 6 days. Similarly, the levels of K^{+} in the apoplastic fluid remained constant (at 24.3 mM) during storage at 5 °C, but decreased only 18% during subsequent storage at 15 °C. The study did not address whether the changes in the pH and mineral levels of the apoplastic fluid resulted from the effect of chilling on membrane permeability or on the ion-exchange capacity of cell walls. However, the data provide evidence against severe membrane dysfunction in chill-injured fruit.

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BIOGRAPHICAL SKETCH

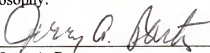
Domingos Almeida was born in Mozambique in 1970 and moved with his family to Portugal in 1976. He obtained a bachelor of science degree in agricultural engineering from the Universidade de Trás-os-Montes e Alto Douro (UTAD), Portugal, in 1993 and a master of science degree in horticulture from the same university in 1995. For three years he taught laboratory courses in general horticulture and vegetable production at UTAD and lectured a course in crop production at Escola Superior de Biotecnologia, Portugal. This experience was determining in his decision to pursue an academic career. In 1996 he was awarded a NATO science fellowship to pursue a doctoral degree at the University of Florida.

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Jerry A. Bartz
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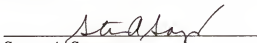
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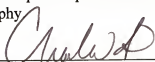
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy

December 1999



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